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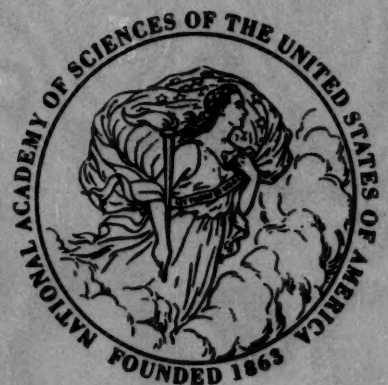

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*A COENZYME CONTAINING PSEUDOVITAMIN B_{12}**

BY H. A. BARKER, H. WEISSBACH,† AND R. D. SMYTH

DEPARTMENT OF AGRICULTURAL BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY

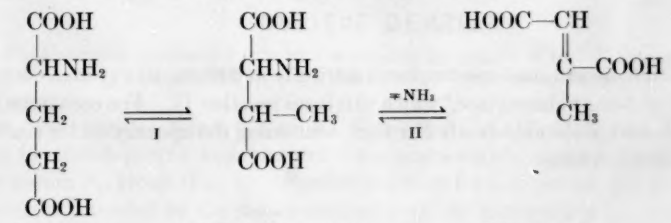
Communicated August 29, 1958

Vitamins of the B_{12} (cyanocobalamin) group apparently participate in several metabolic processes.[1] For example, Helleiner and Woods[2] reported that a cell-free extract of an *Escherichia coli* mutant, which requires either methionine or vitamin B_{12} for growth, forms methionine more rapidly in a reaction mixture containing homocysteine, serine, and adenosine triphosphate when supplemented with vitamin B_{12}. This indicates that the vitamin participates at some point in methyl group formation or transfer. More recently, Wagle *et al.*[3] found that vitamin B_{12} stimulates incorporation of amino acids into the proteins of a microsome-soluble protein system of vitamin B_{12}-deficient rats. Inhibition experiments with an unspecified anti-B_{12} compound indicated that the vitamin is involved in the activation of amino acids. Evidence for the involvement of vitamin B_{12} in the synthesis of deoxyribose by *Lactobacillus leichmanii*[4, 5] and in the reduction of dithio groups[6, 7] has also been presented.

Although these and other studies[1] have identified several metabolic processes in which the cobalamins participate directly or indirectly, the precise roles of these compounds have not been established. No specific enzymatic function has been demonstrated, and no coenzyme form of the vitamin has been described.

We wish to report the isolation of a coenzyme form of pseudovitamin B_{12} which is required for the decomposition of glutamate by particle-free extracts of *Clostridium tetanomorphum*. The coenzyme is involved in the rearrangement of the carbon skeleton of glutamate to form a branched chain amino acid, β-methylaspartate.

Previous studies in this laboratory[8] have shown that the fermentation of glutamate by *C. tetanomorphum* occurs by a sequence of reactions that is entirely different from the tricarboxylic acid cycle. A key compound in this sequence is mesaconate, a branched-chain C_5 dicarboxylic acid. The reversible conversion of glutamate to mesaconate and ammonia involves the following two reactions:



Glutamate β-Methylaspartate Mesaconate

Reaction I has been shown to require a coenzyme that can be removed from crude extracts by treatment with charcoal.[8a] Such extracts can be reactivated by the addition of boiled extract or purified coenzyme (Table 1). Estimation of the rate of reaction I and of coenzyme concentration is based upon the rate of mesacon-

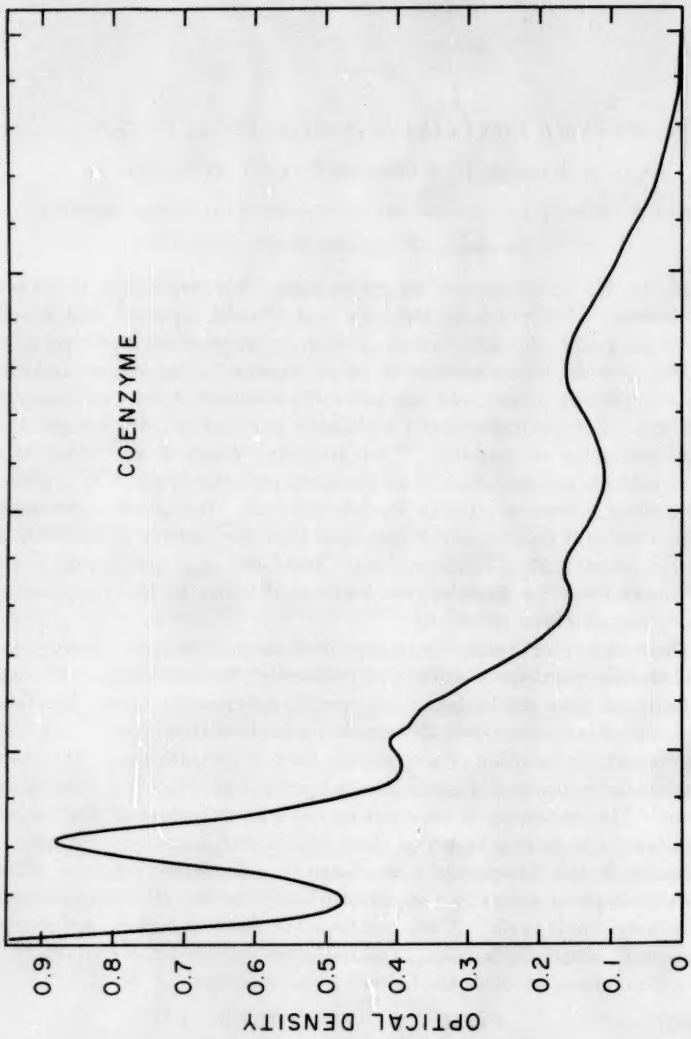


FIG. 1.—Absorption spectrum of the active coenzyme, 1.8×10^{-5} *M*, in 0.03 *M* sodium acetate pH 6.7.

ate formation, determined spectrophotometrically at 240 mμ, in a system containing an excess of β-methylaspartase,[9] which catalyzes reaction II. For coenzyme assay, a charcoal- and protamine-treated extract, containing the apoenzyme for reaction I, is also added in excess.

We have developed a method for the purification of the coenzyme in micromolar amounts from *C. tetanomorphum* by the use of columns of alumina, Dowex-1, and Dowex-50. Fractions containing the most highly purified coenzyme are yellow-orange in color and show a distinctive absorption spectrum (Fig. 1), with maxima

TABLE 1*

COENZYME REQUIREMENT FOR CONVERSION OF GLUTAMATE TO MESACONATE

Expt.	Conditions	ΔO.D. 240 mμ/min
I	Crude extract, 0.2 ml.	0.180
	Charcoal-treated extract, 0.2 ml.	0.008
	Charcoal-treated extract, 0.2 ml. + heated extract, 0.2 ml.	0.142
II	Enzyme	0.002
	Enzyme + 0.02 ml. coenzyme solution	0.035
	Enzyme + 0.04 ml. coenzyme solution	0.076
	Enzyme + 0.08 ml. coenzyme solution	0.118

* All reaction mixtures contained Tris-chloride buffer pH 8.2 0.05 *M*, KCl 0.01 *M*, $MgCl_2$ 0.001 *M*, and potassium L-glutamate 0.01 *M* in a total volume of 1 ml. In Expt. I the crude extract contained 30 mg. protein per ml., and the charcoal-treated extract contained 28 mg. protein per ml.; the heated extract was prepared from the crude extract by heating for 10 minutes at 90° C. In Expt. II the enzyme was 0.05 ml. of a charcoal- and protamine-treated extract containing 23 mg. protein per ml.; the purified coenzyme solution was 9.1×10^{-6} *M* based on the assumption that the extinction coefficient in 0.1 *M* KCN at 367 mμ is the same as for vitamin B_{12}.

at 263, 303, 374, and 457 mμ. When a solution of the coenzyme is exposed to light from a tungsten lamp, the color changes gradually from yellow-orange to red, and the absorption spectrum is modified to give maxima at 261, 351, 407, 495, and 525 mμ (Fig. 2). The latter spectrum is similar to that of vitamin B_{12} and related compounds.[10] The change in the spectrum accompanies inactivation of the co-

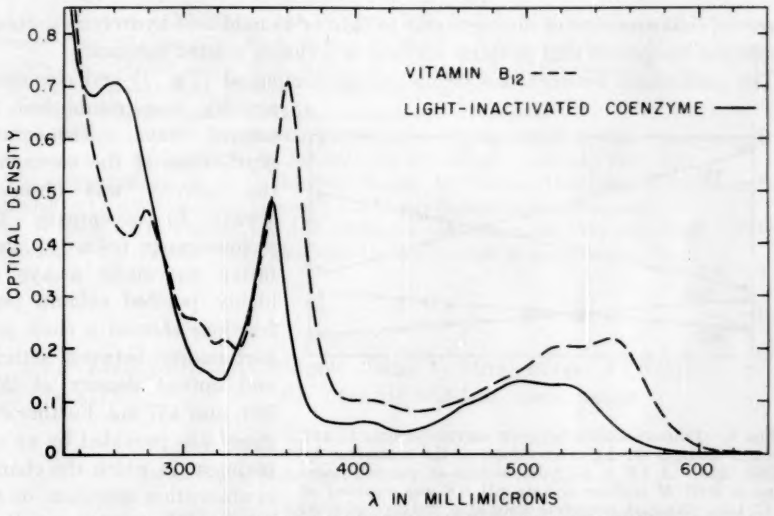


FIG. 2.—Absorption spectra of 2.6×10^{-5} *M* vitamin B_{12} and 1.8×10^{-5} *M* coenzyme inactivated by exposure to a 100-watt tungsten lamp for 105 minutes.

enzyme (see below). When 0.1 *M* KCN is added to the coenzyme, the color changes to reddish-purple, and the spectrum is characteristic of the dicyanide form[10] of the vitamin B_{12} group (Fig. 3). Further evidence for a structural similarity with this group is provided by the demonstration that the coenzyme contains approximately equimolar amounts of cobalt[11] and cyanide. Adenine, ribose, ammonia, and

phosphate are released from the coenzyme by acid hydrolysis, indicating that it is a derivative of pseudovitamin B_{12}.[12] A conspicuous difference between the spectra of the vitamin B_{12} group and the coenzyme is the relatively higher absorption of the latter in the 263–276 mμ region. This indicates that the coenzyme contains one or more additional light-absorbing groups. In confirmation of this conclusion we have

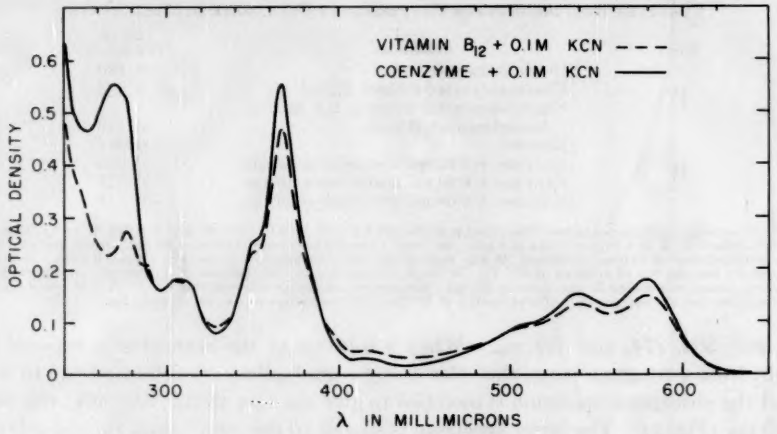


FIG. 3.—Absorption spectra of 1.5×10^{-5} *M* vitamin B_{12} and 1.8×10^{-5} *M* coenzyme in 0.1 *M* KCN.

observed that exposure of the coenzyme to light or to mild acid hydrolysis splits off a colorless compound that contains adenine or a closely related substance.

The connection between the yellow-orange compound (Fig. 1) and coenzyme activity was established in several ways. Throughout purification of the coenzyme, the activity was observed always to accompany the yellow-orange color. Quantitative enzymatic assays on highly purified column peak fractions showed a close proportionality between activity and optical density at 263, 303, and 457 mμ. Further evidence was provided by an experiment in which the change in absorption spectrum, on exposure of the coenzyme to light for various intervals, was correlated with enzymatic ac-

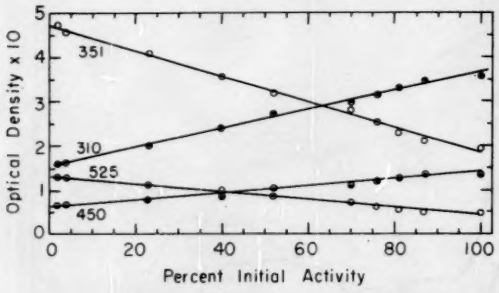


FIG. 4.—Proportionality between enzymatic activity and spectral changes during inactivation of the coenzyme by visible light. A 1.8×10^{-5} *M* solution of purified coenzyme in 0.03 *M* sodium acetate pH 6.8 was exposed at 0° C. to a 100-watt tungsten lamp at a distance of 6–30 cm. At intervals during 105 minutes, samples were removed for determining coenzyme activity, and the spectrum by means of a Cary spectrophotometer. The indicated wave lengths are expressed in millimicrons.

tivity. The decline in activity was found to be directly proportional to the change in optical density at several different wave lengths (Fig. 4). These observations indicate that coenzyme activity and the spectrum belong to the same compound. Neither vitamin B_{12} nor pseudovitamin B_{12}[12] is active in the enzymatic reactions.

We conclude that the coenzyme is a derivative of pseudovitamin B_{12} containing one or more additional purine moieties. The light-sensitivity of the coenzyme and the large shift of the peak in the visible region to longer wave lengths on exposure to light suggest that a functionally important substituent group is attached both to cobalt and to some part of the conjugated double-bond system of the porphyrin-like structure.

The precise role of the coenzyme in the interconversion of glutamate and β-methylaspartate is not yet known.

* This investigation was supported in part by a research grant (E-563) from the National Institutes of Health, Public Health Service, and by a research contract with the Atomic Energy Commission.

† Investigator, National Heart Institute, National Institutes of Health.

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*ON THE REGULATION OF FATTY ACID BIOSYNTHESIS BY LIPOGENIN**

BY G. N. CATRAVAS AND H. S. ANKER

DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CHICAGO

Communicated by William Bloom, September 2, 1958

We have shown[1] that a substance could be extracted from liver or yeast which, in catalytic amounts, markedly increased the rate of fatty acid synthesis in cell-free preparations from fasted rats. We now propose the name of "lipogenin" for this substance. The lipogenin used in these experiments was prepared from yeast

and purified as previously described[2]; it apparently contained yeast mannan, from which the active component could not be separated. Maximal activity, as measured by the incorporation of acetate carbon into the fatty acids in vitro, was observed with 6–10 μg. of lipogenin per gram of liver homogenate.

We wish to report here the results of some experiments with intact animals. The activity of lipogenin was measured by determining the incorporation of labeled acetate in vitro into the liver fatty acids, as previously described.[2] Female Sprague-Dawley rats were fasted for 30 hours and then injected intraperitoneally with 1 mg. of lipogenin in 1 ml. of saline. It was found that the average ratio of acetate incorporation in eight injected and nine control animals was 5:1. The stimulation produced by injection of lipogenin into the intact animal is comparable to that obtained by the addition of lipogenin in vitro to aliquots of liver homogen-

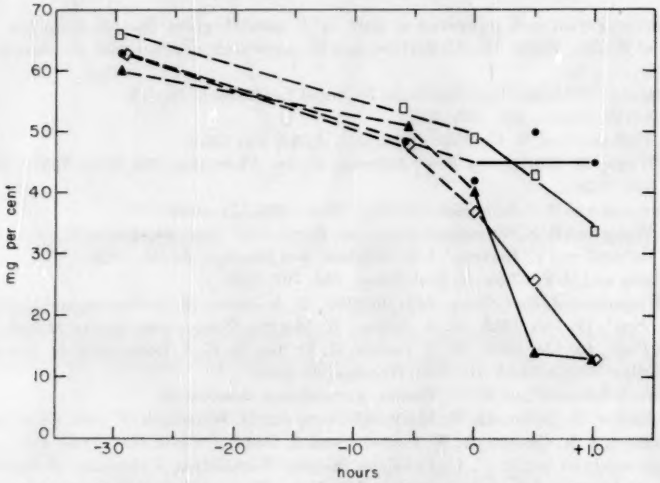

FIG. 1.—Blood glucose concentrations in fasted rats; test materials administered at zero time. ● Saline control (average of 2 experiments). ▲ 12 units of crystalline zinc insulin. □ 1 mg. lipogenin (similar values were obtained in two additional animals). ◇ 5 mg. lipogenin (due to the high viscosity of the blood, the last point on this curve is uncertain).

ates (a ratio of 6:1). The injection of similar amounts of mannan, glycogen, glucose, or 12 units of crystalline zinc insulin was without effect on acetate incorporation. The in vivo effect of lipogenin was observed within 0.5–2.5 hours after injection. Acetate incorporation was even greater in one experiment in which 5 mg. of lipogenin was injected. On the other hand, the injection of lipogenin into a fed rat did not result in any significant rise in acetate incorporation, nor did its in vitro addition to an aliquot of liver homogenate from fed animals increase acetate incorporation.[2]

The injection of 1 mg. of lipogenin into fasted rats had a marked effect on water distribution and on blood glucose concentration. After the injection of 5 mg. of lipogenin, the blood glucose in a fasted animal dropped rapidly, though less sharply than after 12 units of insulin (Fig. 1). The animals given lipogenin

were very thirsty, their limbs became edematous, and blood viscosity was increased. These latter effects were not observed after insulin administration. It is at present not clear whether alteration in the water distribution is due to a direct or an indirect effect of lipogenin or to the presence of mannan. The drop in the blood glucose concentration and the disturbance of the water distribution could be prevented by two intraperitoneal doses of 1.5 ml. of isotonic glucose given together with, and 1 hour after the injection of lipogenin.

While both insulin and lipogenin lower the blood sugar concentration in fasted rats, only the latter increases lipogenesis. This fact suggests that, whereas insulin permits blood glucose to enter the body's cells, lipogenin also causes the conversion of blood glucose into fat. Since this conversion normally proceeds in fed animals, the administration of lipogenin to fed rats does not result in a decrease in the blood glucose, in contrast to the effect of insulin.[3] It is therefore suggested that lipogenin is one of the means of regulation of fatty acid biosynthesis in mammalian organisms.

* Supported by grants from the National Heart Institute, National Institutes of Health, United States Public Health Service.

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*ELECTRON-SPIN RESONANCE STUDIES OF FREE-RADICAL INTERMEDIATES IN OXIDATION-REDUCTION ENZYME SYSTEMS**

BY BARRY COMMONER, BARBARA B. LIPPINCOTT, and JANET V. PASSONNEAU

HENRY SHAW SCHOOL OF BOTANY, WASHINGTON UNIVERSITY, ST. LOUIS, MISSOURI

Communicated by E. U. Condon, September 2, 1958

I. INTRODUCTION

The oxidation-reduction processes that occur in living cells and in systems composed of isolated enzymes involve a succession of reactions in which electron transfer is the common characteristic. This conclusion has been inferred from chemical evidence about the structure and reactivity of the system components, but relatively little is known about the physical processes which govern electron transfer among them. This paper is concerned with one of the unresolved problems: the valence of electron transfer.

Direct chemical evidence indicates that the net effect of many biochemical oxidation-reduction reactions is the transfer of two electrons. In other cases, notably the cytochrome–cytochrome oxidase system, the same type of evidence indicates that the transfer is univalent. The thermodynamic barriers to the necessary coupling of 2-electron and 1-electron processes are formidable, and this problem has been under active consideration for some time.

On the basis of work with model systems, Michaelis proposed a solution which is best summarized in his own statement that "all oxidations of organic molecules, although they are bivalent, proceed in two successive univalent steps, the inter-

mediate state being a free radical."[1] In support of this proposal Michaelis showed that electrometric titration of various organic oxidation-reduction systems exhibits a node which suggests that each of them involves two successive electron transfers. Spectrophotometric studies suggested the presence of intermediates between the fully oxidized state and the fully reduced form of various substances, especially flavins, that participate in biochemical oxidation-reduction processes. Since the fully oxidized and fully reduced forms differ by 2 electrons, the intermediate contains an unpaired electron and is therefore a free radical. The operative conclusion which emerges from Michaelis' work is that free radicals must occur as intermediates in all biochemical oxidation-reduction processes. Following Michaelis, several investigators added to the indirect evidence that favors this hypothesis, but it has also been subjected to a rather critical analysis by Westheimer.[2]

Recently a new experimental approach to the problem has become possible which has the merit of yielding direct physical evidence concerning the occurrence of the unpaired electrons associated with free radicals. The method is based on the phenomenon of electron-spin resonance which may be studied by means of a spectrometer capable of detecting and characterizing the gyromagnetic properties of very small amounts of unpaired electrons (of the order of 10^{-10} moles). The initial efforts to employ this technique to study biological oxidation-reduction processes, reported by Commoner *et al.*,[3] were limited by the fact that the ESR spectrometer then available was not capable of measurements with wet samples. This limitation has now been overcome by the construction (by Professor J. Townsend) of an ESR spectrometer capable of detecting about 10^{-10} moles of unpaired electrons in samples containing about 0.15 ml. of liquid water. With this spectrometer it has been shown that light-induced free radicals occur in chloroplasts and living algae and, in a preliminary way, that free radicals also occur in certain in vitro enzymatic oxidation-reductions.[4] Cursory observations of ESR signals in enzyme systems have also been reported by Ehrenberg and Ludwig and by Beinert.[5]

The present paper reports detailed experiments on a flavoprotein oxidation-reduction enzyme and less detailed studies of a series of common oxidation-reduction enzyme systems. The results confirm Michaelis' proposal that free-radical intermediates are characteristic features of enzymatic electron transport.

II. METHODS

The ESR spectrometer detects the absorption of incident microwave energy (9,000 Mc/sec) which results from energy levels imposed by an external magnetic field on unpaired electrons present in the sample. The gyromagnetic properties of an unpaired electron are such that an external field of about 3,300 gauss will induce absorption of 9,000 Mc/sec incident energy. The spectrometer automatically varies the strength of the external magnetic field over a selected range centered about 3,300 gauss while monitoring the absorption of microwave energy by the sample. The record of a run represents the first derivative of the energy absorbed, plotted against the strength of the external magnetic field. Comparison of the absorption due to a standard preparation of a stable free radical and that yielded by the sample results in a rough approximation of the number of electron spins present in the latter.

A given electron-spin resonance absorption may be characterized in several ways.

(1) The gyromagnetic ratio g is determined from the precise value of the external field at which the center of the ESR absorption occurs, in accordance with the equation: ν = a constant $\times$ g $\times$ H, where ν is the frequency of the incident energy and H is the strength of the external magnetic field. The values of g observed for organic free radicals vary from about 2.000 to 2.007,[6] a given free radical is characterized, but not uniquely, by its g-value. (2) The width of the ESR absorption (usually expressed as the width in gauss, at half the maximum absorption) is indicative of the relaxation processes in which the unpaired electrons are involved. This is a complex, but nevertheless convenient, empirical character. (3) When an unpaired electron is associated with an atomic nucleus that itself possesses a magnetic moment, coupling between the two occurs. This gives rise to a splitting of the energy levels imposed on the electron, so that the ESR absorption then exhibits a characteristic hyperfine structure. In the present work the ESR signals were not sufficiently intense to permit measurements at the high resolving power (and low sensitivity) required to observe hyperfine structure.

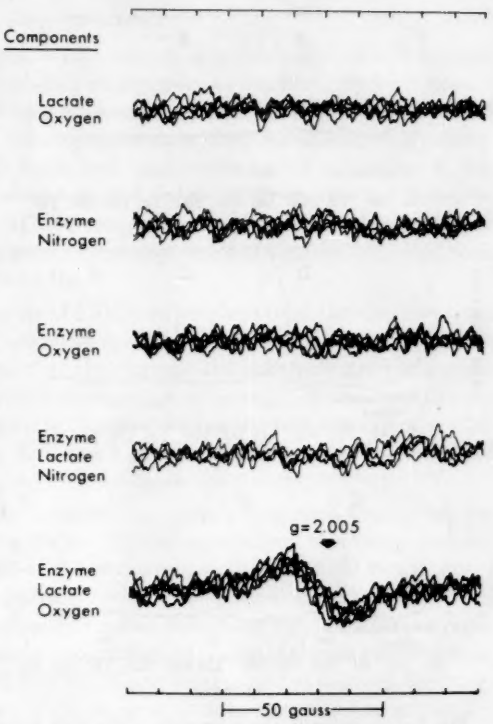

FIG. 1.—Electron spin resonance (ESR) from the separate components of the lactic oxidative decarboxylase system and from the complete system (enzyme plus lactate) in nitrogen and in oxygen: 8 mg. lactic oxidative decarboxylase, 25 mg. *dl*-lactate, and 0.2 ml. PO_4 buffer at pH 5.9. The magnetic field increases toward the right along the abscissa. The ordinate represents the rate of change with respect to field strength of microwave energy (9,000 Mc/sec) absorbed by the sample as the strength of the magnetic field is varied. The records shown are superimposed tracings of four to five sequential runs.

In most experiments 5–50 mg. of the enzyme preparation are placed in an ESR spectrometer cell (a Pyrex cell, 0.5 $\times$ 10 $\times$ 20 mm. internally) and a suitable amount of substrate added in a dry form. About 0.1–0.2 ml. of buffer is then added to the cell, and the contents are mixed rapidly with a stainless-steel wire. In some cases the resulting mixture is a slurry rather than a solution.

The sample cell is inserted into the ESR spectrometer cavity, which is then tuned, and the instrument set to make rapid sweeps across the region of 3,300-gauss external field strength. Each sweep requires a minimum of 90 seconds. The transient character of enzyme intermediates requires that measurements be rapid.

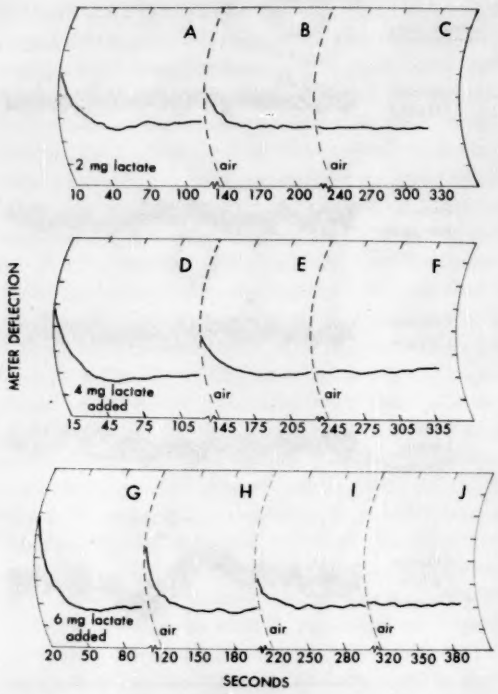

FIG. 2.—Variation in the magnitude of the ESR signal (i.e., the meter deflection at the magnetic field at which this deflection is maximum) with time after the addition of varying amounts of lactate to a sample of 8 mg. of lactic oxidative decarboxylase in 0.2 ml. PO_4 buffer at pH 5.9. The system was saturated with air at the beginning of each run. Curves *A*, *B*, and *C* show the effects of adding 2 mg. of lactate to the enzyme at time zero, and of aeration at 130 and 230 seconds. Curves *D*, *E*, and *F* show the effects of adding 4 mg. of lactate at time zero to the enzyme system after the completion of run *C*. The system was aerated at 135 and 235 seconds. Curves *G*, *H*, *I*, and *J* show the effects of adding 6 mg. of lactate to the system at time zero, after the completion of curve *F*, with aeration at 110, 210, and 310 seconds.

Under these conditions inherent noise is a significant factor, and it becomes necessary to distinguish between meter deflections due to noise and those due to an ESR signal. This may be accomplished by superimposing, by tracing, the records of successive runs; random deflections due to noise cancel out, and the deflections due to the signal coincide.

During the course of the experiments reported in this paper it was discovered that exposure of solutions of flavoprotein enzymes to moderate intensities of visible light induces the formation of free radicals. Accordingly, in most cases, the data reported below refer to systems which were protected from direct exposure to light. This problem is discussed in detail in another paper.[7]

III. MATERIALS

Enzyme preparations used in the experiments described below were derived from the following sources. Cytochrome C reductase was isolated and purified according to the method of Mahler *et al.*[8] Cytochrome oxidase was isolated and purified according to the method of Wainio *et al.*[9] The SC factor was prepared according to the method of Clark *et al.*[10] Lactic oxidative decarboxylase, a crystalline preparation, was isolated from *Mycobacterium phlei* and was kindly supplied by Dr. William B. Sutton. D-α-amino acid oxidase, purified, and old yellow enzyme, purified, were obtained from Mann Research Laboratories. Aldolase, crystalline, and glyceraldehyde phosphate dehydrogenase, crystalline, were obtained from Worthington Biochemical Company. Alcohol dehydrogenase (yeast), purified; lactic acid dehydrogenase, purified; glucose-6-phosphate dehydrogenase, purified; and cytochrome C, crystalline, were obtained from Sigma Chemical Company. Succinic acid dehydrogenase, purified, was obtained from Delta Chemical Company.

IV. RESULTS

1. *Lactic Oxidative Decarboxylase.*—This enzyme was selected for detailed study. It has been isolated, purified, and studied with respect to biochemistry by Sutton.[11] We are indebted to Dr. Sutton for very generous supplies of extensively purified and recrystallized enzyme. Sutton's investigations show that this enzyme will rapidly dehydrogenate and decarboxylate lactic acid in the presence of molecular O_2, the products being carbon dioxide, acetic acid, and water. The enzyme is a flavoprotein and contains 2 molecules of FMN per enzyme molecule. Sutton has suggested that the catalytic activity of the enzyme involves a series of electron transfers, with a free radical occurring as an intermediate.[12]

Figure 1 shows the results of a series of ESR runs obtained from the enzyme alone, from lactate alone, and from the complete system. Neither the enzyme itself nor the lactate exhibits an ESR signal. If the enzyme and substrate are present together but under anaerobic conditions, no signal is detected. However, if oxygen is also present, a distinct ESR signal is observed during the first few minutes after the components are mixed. The ESR has a g-value of 2.005 and a half-width of about 13 gauss.

The effects of variation in lactate concentration and in oxygen tension on the size of the ESR are shown in Figure 2. In this experiment the spectrometer's magnetic field is set at the position of the resonance, so that the ESR signal causes only a simple deflection of the indicating meter. The recording chart is employed to register the changes, with time, in the meter deflection. By this means rapid changes in ESR magnitude can be followed during the course of the enzyme reaction.

When 2 mg. of lactate are added to 8 mg. of previously aerated enzyme, the magnitude of the ESR signal declines rapidly as soon as measurements begin, and the signal disappears within 40 seconds (curve *A*). After the signal declines to zero, aeration of the sample restores the signal to a degree (curve *B*). The meter deflection again falls to zero in 20 seconds. Curve *C* shows that an additional aeration now fails to restore the signal. These results show that first oxygen and then lactate are rapidly depleted in the sample cell and limit the size of even the initial signal that is observed.

Curve *D* shows that enzyme activity and the ESR signal can be restored in a system depleted of lactate by the addition of this substrate. Again the signal declines with time. Curve *E* shows that this effect is due in part to depletion of oxygen, but finally lactate is also lacking (curve *F*). Curve *G* and those following show the effects of a second addition of 6 mg. of lactate and of subsequent aeration. As expected, the addition of the larger amount of lactate prolongs the time during which the system continues to respond to the presence of oxygen.

This experiment shows that the magnitude of the ESR signal is a function of the lactate and oxygen concentrations and that a single sample of enzyme exhibits an ESR in a succession of reactions. It will be noted that the earliest observed signals were obtained about 10–12 seconds after the system components are mixed—i.e., the time required to tune the spectrometer. However, it is possible to estimate the size of the true initial signal and its relation to the amount of lactate added to the system. In Figure 3, curves *A* and *D* and *G* of Figure 2 are plotted semilogarithmically. The signal size declines exponentially, so that extrapolation to

zero time (the time at which the lactate is added to the sample) is possible. This intercept is a measure of the true initial signal value.

From a series of such experiments it is possible to describe the relationship between lactate concentration and the true initial size of the ESR signal. The results are shown in Figure 4. Up to a concentration of 6 mg. of lactate per 0.2 ml. of solution (containing 8 mg. of crystalline lactic oxidative decarboxylase) the size of the ESR signal is proportional to the lactate concentration. Beyond this point

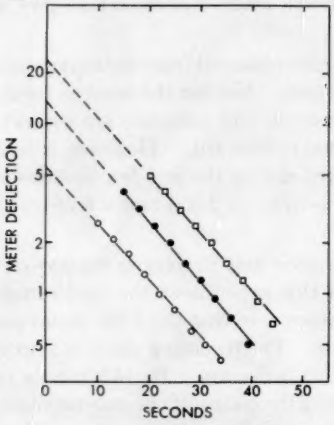

FIG. 3.—Semilogarithmic plots of the meter deflections from Fig. 2 against time. Open circles represent curve *A* of Fig. 2; solid circles, curve *D*; and open squares, curve *G*. The broken lines are extrapolations of the curves to zero time (the time at which the lactate is added to the sample). This intercept represents the initial meter deflection.

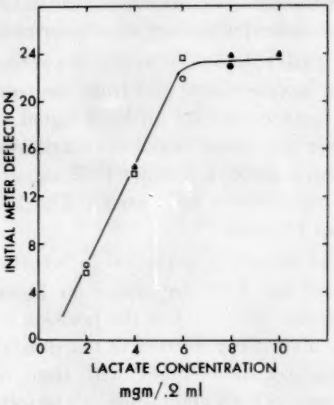

FIG. 4.—The relation of lactate concentration to the initial meter deflection (obtained by extrapolating data similar to that in Fig. 2 to zero time). Circles, squares, and triangles represent experiments carried out on different days using different preparations of enzyme. Open symbols represent sequential additions of lactate to a single sample of enzyme. Closed symbols represent a single addition of lactate to a sample of enzyme.

the initial ESR signal remains constant despite increasing lactate concentration The experimental values extrapolate to zero ESR signal near zero lactate concentration. Data derived from three separate experiments, carried out at different times, show an excellent quantitative agreement.

A series of similar experiments in which the enzyme concentration is varied in the presence of 6 mg. of lactate gives the results shown in Figure 5. With considerable precision, the magnitude of the initial ESR signal at the time of mixing is proportional to the enzyme concentration and extrapolates to zero near zero enzyme concentration.

Figure 6 shows the effects of two inhibitors of the lactic oxidative decarboxylase—pyruvate (which competes with lactate for the enzyme's active sites) and *p*-chloromercuribenzoate (PCMB), which appears to inactivate essential -SH groups.[13] The control curve of Figure 6 shows that if excess lactate is added to the enzyme in the absence of inhibitor, the ESR signal appears and is maintained for a period of 10 minutes. If the same amount of lactate is added to the enzyme after a previous

addition of pyruvate (30 mg. in 0.15 ml.), no ESR signal appears. Thus, in the presence of a competitive concentration of pyruvate, the enzyme fails to act on lactate and shows no ESR signal. PCMB has a similar effect. In this case the inhibitor itself, on addition to the enzyme, generates a free radical, possibly because it brings the -SH group into its half-oxidized state. However, when lactate is added, the signal fails to increase in size.

It can be shown that the failure of the enzyme to respond to lactate when these inhibitors are present is not due to inactivation of the enzyme's prosthetic group. We have found that flavins, either free or in the form of enzyme prosthetic groups, will respond to visible light by forming a free radical apparently identical with that which occurs as an oxidation-reduction intermediate.[7] If lactic oxidative decarboxylase is illuminated in the presence of either PCMB or pyruvate after the addition of lactate has failed to elicit an ESR signal, light has its expected effect, and a substantial new ESR is observed (Fig. 6). This shows that, despite the presence of the inhibitor, which prevents the enzyme's response to lactate, the FMN prosthetic group is still capable of forming a free radical. This result is expected from the fact that these inhibitors act only on the protein moiety of the enzyme.

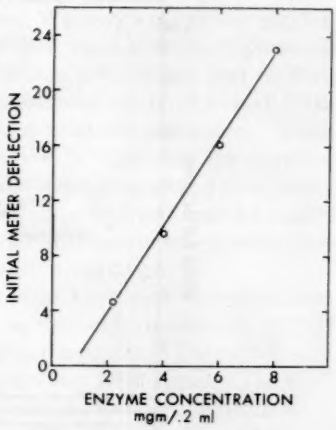

FIG. 5.—The relation between concentration of lactic oxidative decarboxylase and initial meter deflection (obtained by extrapolation to zero time of data similar to that in Fig. 2). In all cases 6 mg. of lactate was introduced at time zero. The point at 8-mg. enzyme is the average of two experiments, while the other three points represent single experiments.

The foregoing results confirm Sutton's proposal that the activity of the lactic oxidative decarboxylase proceeds through a free-radical intermediate. Qualitatively, the experiments demonstrate an obligate relationship between enzyme activity and the presence of the free radical. The quantitative results also conform with the expectation that the free radical is an intermediate which achieves a steady-state concentration that depends on the rate of enzyme activity. The turnover number of lactic oxidative decarboxylase, calculated by Sutton from the rate of enzymatic activity, is about 2,000. On this basis the enzyme system described in Figure 3 under optimum conditions can convert 2 mg. lactate and return to a zero rate of electron transport in 20 seconds; 4 mg. can be converted in 40 seconds and 6 mg. in 60 seconds. Figure 3 shows that in the three cases the times during which an ESR is detectable are 50, 65, and 80 seconds, respectively. This agreement shows that the free radical is present only during the course of enzymatic electron transfer.

The quantitative relationships between the size of the ESR and the enzyme and lactate concentration also support Sutton's proposal. Like the rate of enzyme activity, the size of the ESR is proportional to the enzyme concentration (Fig. 5). The curve relating lactate concentration to size of initial ESR (Fig. 4) resembles the Michaelis-Menten function, which relates the rate of enzyme activity to substrate concentration.

So long as an inhibitor prevents lactate from being bound by the enzyme or from transmitting electrons to FMN, the free radical is absent. Substitution of another source of electrons, through the effect of light, induces an ESR which is not distinguishable from that found during ordinary enzyme activity. This suggests that the ESR observed when the enzyme is active is probably due chiefly to an FMN free radical.

In sum, all the foregoing results support the conclusion that electron transfer mediated by lactic oxidative decarboxylase gives rise to a free-radical intermediate probably derived from the enzyme's prosthetic group and that the steady-state concentration of free radical varies with the rate of electron transport.

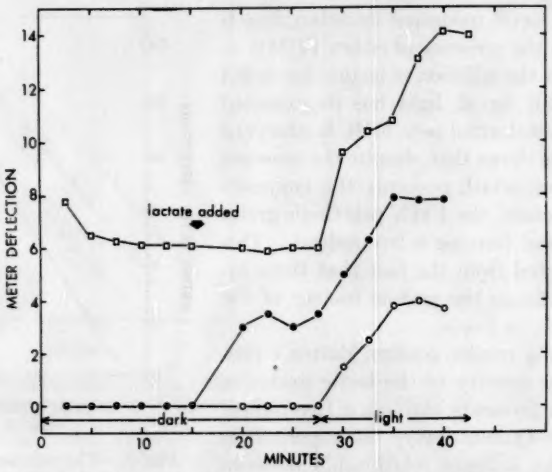

FIG. 6.—The effect of inhibitors and light on the electron-spin resonance obtained from lactic oxidative decarboxylase. The maximum meter deflection is plotted against time. In all cases the system contained 8 mg. of enzyme and 0.15 ml. of phosphate buffer at pH 5.9. The closed circles represent a control experiment in the absence of inhibitor. In the experiment represented by the open circles 30 mg. of pyruvate were present. In the experiment represented by the open squares 5 mg. of *p*-chloromercuribenzoate were present. The arrow indicates the time at which 30 mg. of lactate were added to all three samples.

2. *Other Enzymes.*—ESR results obtained from a number of enzyme systems are summarized in Table 1. The enzymes studied include 5 dehydrogenases, 5 flavoprotein enzymes, and the cytochrome C–cytochrome oxidase system.

All the experiments with dehydrogenases (alcohol, lactic acid, glucose-6-phosphate, 3-phosphoglyceric acid, and succinic) show that an ESR appears during the activity of the complete system consisting of the substrate, the enzyme, and the appropriate coenzyme. No resonances were observed in any of the separate components. The 3-phosphoglyceraldehyde dehydrogenase was also studied in a more complex system, in which aldolase and fructose 1,6-diphosphate was present to generate the necessary 3-phosphoglyceraldehyde. Again an ESR was observed only when the system included the enzymatically formed substrate, dehydrogenase, and

DPN^+. This result shows, incidentally, that the activity of aldolase, an enzyme which does not appear to mediate electron transfer, is not accompanied by free-radical formation. Results obtained with alcohol dehydrogenase and with glyceraldehyde phosphate dehydrogenase show that the activity of this enzyme leads to an ESR signal whether the process is in the "forward" direction (i.e., coupled oxidation-reduction of alcohol or glyceraldehyde phosphate and DPN^+) or in the "reverse" direction (coupled oxidation-reduction of acetaldehyde or phosphoglyceric acid and DPNH).

The experiments with lactic oxidative decarboxylase that have already been described offer the most detailed evidence regarding the occurrence of free radicals during the activity of a flavoprotein enzyme. Three other flavoproteins have been studied in a preliminary way: D-amino acid oxidase, Warburg's old yellow enzyme, and cytochrome reductase. Unlike the decarboxylase, none of these preparations was crystalline. Commercial preparations of D-amino acid oxidase and of Warburg's old yellow enzyme in the absence of their substrates exhibited a small ESR, which showed a significant transitory increase when substrate was added. These are the only instances in which an ESR was observed in other than the complete enzyme system. As shown elsewhere, when flavoproteins are exposed to ordinary room light, free radicals which may be quite stable in some conditions are readily produced.[7] It is possible that the signals observed in these enzyme preparations were due to exposure to light at some stage during their preparation.

Results of experiments with cytochrome reductase have been described elsewhere.[14] This enzyme is a flavoprotein which is reversibly oxidizable, DPNH serving as electron donor and cytochrome C as electron acceptor. The reduction of the reductase by DPNH is accompanied by a transitory ESR signal. The ESR is enhanced if cytochrome C is then added. The reduced form of the reductase is then reoxidized, and the cytochrome C is reduced. When this signal itself becomes diminished with time, the addition of cytochrome oxidase (which reoxidizes reduced cytochrome C) regenerates an intensified ESR signal. The presence of atabrine, an inhibitor of cytochrome reductase, causes the ESR exhibited by the DPNH–reductase–cytochrome C system to disappear. These results show that the enzymatic activity of the several flavoproteins studied is uniformly associated with an ESR.

The electron transfers mediated by the cytochrome–cytochrome oxidase system are necessarily univalent, since they involve a univalent change in the state of an iron atom. Results summarized in Table 1 show that this transfer is associated with an ESR. Oxidized cytochrome C does not exhibit an ESR. When cytochrome C is reduced with ascorbate, a small signal is observed. This may be due to some ascorbate free radical which is easily formed and stable in neutral solution. When cytochrome oxidase, which itself lacks an ESR, is added to the reduced cytochrome C and O_2 is present, an intensified signal appears. The presence of cyanide, which inhibits cytochrome oxidase specifically, prevents this effect.

The ESR signals described in Table 1 are not sufficiently intense to permit a search for hyperfine structure. However, the observed g-values and line widths fall within the range of corresponding values observed in a number of organic free radicals.[6]

In the case of the flavoprotein enzymes, it can be concluded that the observed ESR is due, at least in part, to a free-radical form of the flavin prosthetic group.

TABLE 1

ENZYME	SYSTEM COMPONENTS	ELECTRON-SPIN RESONANCE: g-Value	ELECTRON-SPIN RESONANCE: Half-width (Gauss)	APPROXIMATE MOLES OF FREE RADICAL DETECTED × 10^{-10}
Lactic oxidative decarboxylase	Complete: enzyme (8 mg.), *dl*-lactate (10 mg.), O_2	2.005	13	4
	Enzyme only	...	...	...
	dl-Lactate only	...	...	...
	Enzyme, lactate in N_2	...	...	...
D-α-amino acid oxidase	Complete: enzyme (50 mg.), alanine (20 mg.), O_2	2.002	15	3
	Enzyme only	2.002	15	2
	Alanine only	...	...	...
Warburg's old yellow enzyme	Complete: enzyme (50 mg.), TPNH (5 mg.), O_2	2.003	14	5
	Enzyme only	2.003	14	2
	TPNH only	...	...	...
Glyceraldehyde phosphate dehydrogenase	Complete: enzyme (10 mg.), 3-phosphoglyceric acid (30 mg.), DPNH (10 mg.)	2.001	13	1
	Enzyme only	...	...	...
	Phosphoglyceric acid only	...	...	...
	DPNH only	...	...	...
	Complete: enzyme (10 mg.), aldolase (10 mg.), fructose-1,6-diphosphate (30 mg.), DPN^+ (10 mg.)	2.001	13	1
	Aldolase only	...	...	...
	Fructose-1,6-diphosphate only	...	...	...
	DPN^+ only	...	...	...
	Aldolase, fructose-1,6-diphosphate, DPN^+	...	...	...
Alcohol dehydrogenase	Complete: enzyme (50 mg.), ethyl alcohol (50 per cent, 0.06 ml.), DPN^+ (30 mg.)	2.006	13	1
	Enzyme, DPN^+	...	...	...
	Enzyme only	...	...	...
	Alcohol only	...	...	...
	DPN^+ only	...	...	...
	Complete: enzyme (50 mg.), acetaldehyde (0.1 ml.), DPNH (30 mg.)	2.006	13	1
	Enzyme, DPNH	...	...	...
	DPNH only	...	...	...
	Acetaldehyde only	...	...	...
Glucose-6-phosphate dehydrogenase	Complete: enzyme (30 mg.), glucose-6-phosphate (10 mg.), TPN^+ (10 mg.)	2.007	18	1
	Enzyme only	...	...	...
	Glucose-6-phosphate only	...	...	...
	TPN^+ only	...	...	...
	Enzyme, TPN^+	...	...	...
DPNH–cytochrome C reductase	Enzyme (30 mg.), DPNH (8 mg.)	2.006	18	2
	DPNH only	...	...	...
	Complete: enzyme (30 mg.), DPNH (8 mg.), cytochrome C (30 mg.)	2.006	18	2
	Cytochrome C only	...	...	...
	Complete + inhibitor: enzyme, DPNH, cytochrome C, atabrine (30 mg.)	...	...	...
	Complete + oxidase: enzyme (30 mg.), DPNH (8 mg.), cytochrome C (30 mg.), cytochrome C oxidase (30 mg.)	2.006*–2.004	18*–11	3

TABLE 1—*Continued*

Enzyme	System Components	Electron-Spin Resonance: g-Value	Electron-Spin Resonance: Half-width (Gauss)	Approximate Moles of Free Radical Detected × 10^{-10}
Cytochrome C oxidase	Complete: enzyme (50 mg.), reduced cytochrome C (40 mg.), O_2	2.004	11	4
	Enzyme only	...	...	...
	Reduced cytochrome C only	2.004	11	2
	Complete + inhibitor: enzyme, reduced cytochrome C, KCN (5 mg.), O_2	2.004	11	2
	Enzyme, KCN	...	...	...
Succinic dehydrogenase	Complete: enzyme (10 mg.) succinate (10 mg.), cytochrome C (10 mg.), SC factor (0.03 ml.)	2.007	16	3
	SC factor only	2.007	16	<1†
	SC factor, succinate	2.007	16	2
	Enzyme, SC factor, succinate	2.007	16	2.5
	Enzyme only	...	...	...
	Succinate only	...	...	...
	Complete + oxidase: SC factor, succinate, enzyme, cytochrome C, cytochrome C oxidase	2.007	16	4

* Upon the addition of cytochrome oxidase to the complete cytochrome reductase system, the g-value of the resonance begins a systematic shift from $g = 2.006$ to $g = 2.004$ and the half-width shifts gradually from 18 to 11 gauss. The changes are those expected in a shift from a resonance due to the cytochrome reductase system to a resonance due to the cytochrome oxidase system.

† The resonance due to the SC factor only is extremely small compared to the noise present and may not represent a significant ESR.

When flavoprotein enzymes are illuminated in the absence of both electron donor and acceptor, an ESR is observed which appears to be similar to that produced during enzymatic activity. In the case of dehydrogenases it is not known at present to what degree DPN and TPN free radicals and possible free-radical forms of the substrates contribute to the observed ESR's. Similarly, the precise molecular origin of the ESR observed in the cytochrome–cytochrome oxidase system cannot be specified as yet. However, in all cases the observed ESR's are due to free radicals which are present only during the course of enzyme activity.

V. CONCLUSIONS

The foregoing experiments confirm both the specific and the generic aspects of Michaelis' hypothesis.

Detailed study of lactic oxidative decarboxylase shows that enzyme activity is invariably associated with a free radical, probably due to the FMN prosthetic group. When the rate of enzyme activity is varied by regulating the substrate and enzyme concentrations, the steady-state concentration of free radical varies proportionally. This behavior is expected of an oxidation-reduction intermediate. The effects of inhibitors on the free-radical level are equally characteristic of such an intermediate. The data confirm Sutton's specific proposal that the reactions mediated by this enzyme include at least one intermediate which has a free-radical form.

The survey of a series of oxidation-reduction enzymes shows that in all cases free radicals occur during the course of activity. The enzymes studied are of sufficient variety and occur so widely among living cells as to give substantial support to Michaelis' proposal that all biological transport is mediated by free-radical intermediates.

* This work was supported in part by the Office of Naval Research, by research grant C-3983 from the National Cancer Institute, United States Public Health Service, and by the United States Air Force through the Air Force Office of Scientific Research of the Air Research and Development Command.

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*LIGHT-INDUCED FREE RADICALS IN FMN AND FLAVOPROTEIN ENZYMES**

BY BARRY COMMONER AND BARBARA B. LIPPINCOTT

HENRY SHAW SCHOOL OF BOTANY, WASHINGTON UNIVERSITY, ST. LOUIS, MISSOURI

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I. *Introduction.*—There is some evidence that visible light may influence the rate of cellular respiration[1] and of isolated oxidation-reduction enzyme systems.[2, 3] The evidence is not sufficiently extensive to indicate whether the effect is a general one, nor is it sufficiently detailed to suggest a possible mechanism.

The experiments reported below provide evidence on these questions. They show that flavin prosthetic groups and flavoprotein oxidation-reduction enzymes readily form free radicals when exposed to ordinary intensities of visible light. The light-induced free radicals appear to be identical with those ordinarily formed as intermediates in the oxidation-reduction process carried out by these components. This effect provides a mechanism which can mediate the influence of light on cellular oxidation-reduction. Since flavoprotein enzymes participate in a wide range of metabolic systems, the evidence also indicates that the observed effects of light on cellular processes is probably of quite general significance.

II. *Methods.*—In the experiments reported below, free radicals were detected and characterized by the electron-spin resonance technique. The electron-spin resonance spectrometer employed and the general features of the technique have been described in previous papers.[4, 5] The properties of the spectrometer that are of particular interest in the present work are (*a*) that the sample may contain as much

as 0.15 ml. of liquid water and (*b*) that the resonance cavity has a slotted end which permits illumination of the sample during ESR measurements. To illuminate the sample, light from a 750-watt tungsten lamp was condensed, cooled by a $CuSO_4$ filter, and directed through the slotted end of the cavity onto the Pyrex sample cell.

III. *Results.*—When a 10^{-2} *M* solution of flavin mononucleotide (FMN) in pH 7 phosphate buffer is examined in the dark in the ESR spectrometer, no signal is observed; free radicals are therefore undetectable (see Fig. 1). However, immediately upon illumination, an ESR signal is readily detected. The number of unpaired electrons detected by the spectrometer can be estimated from the size of the signal. Such an estimation indicates that, on illumination, about 1 per cent of the FMN present is in the free-radical form. The signal is sufficiently intense to permit recording under conditions of high resolution (which tend to reduce the

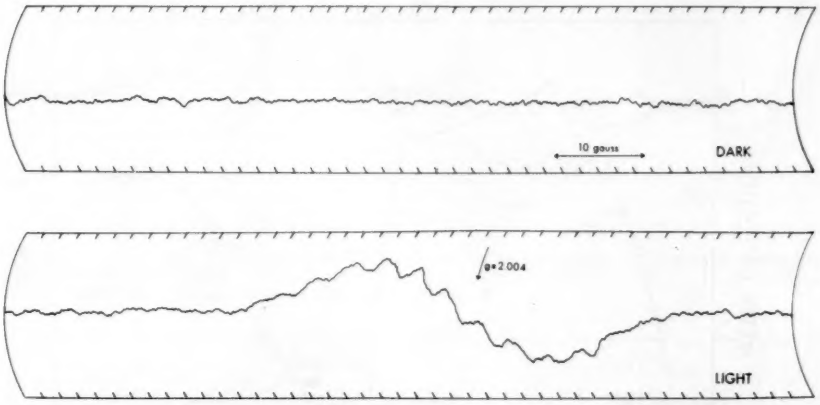

FIG. 1.—Electron-spin resonance (ESR) obtained from a 10^{-2} *M* solution of FMN in pH 7 phosphate buffer in the dark (*upper curve*) and in the light (*lower curve*). Magnetic field increases toward the right along the abscissa. The ordinate is the meter deflection, representing the rate of change, with respect to field strength, of the absorption of 9000 Mc/sec microwave energy by the sample as the field strength is varied automatically. The curves represent the derivatives of absorption curves.

spectrometer's sensitivity). Such a highly resolved signal, reproduced in Figure 1, shows a characteristic hyperfine splitting, consisting of thirteen subpeaks uniformly spaced at about 4 gauss apart.

The complexity of this pattern indicates that the free radical's unpaired electron is not highly localized in the molecule and that the electron-spin resonance due to the unpaired electron is split by magnetic interaction with several atomic nuclei. Of the atoms present in the FMN molecule, only nitrogen and hydrogen possess nuclear spins and are hence capable of such interaction. The over-all width of the resonance is about 50 gauss. In aromatic free radicals, in which the unpaired electron interacts solely with protons, the maximum observed resonance width is about 30 gauss.[6] Hence, in the FMN free radical, the unpaired electron must be affected largely by interaction with nitrogen atoms, although a lesser interaction with protons is not excluded.

When a neutral solution of FMN is illuminated, the yellow color characteristic of the oxidized state fades, and a greenish color develops. On illumination in the absence of oxygen, the absorption maximum at 375 mμ shifts to 320 mμ, while the absorption maximum at 450 mμ nearly disappears. If the illuminated solution is then aerated, the original absorption spectrum and yellow color characteristic of oxidized FMN are restored. Figure 2 shows the effect of oxygen on the ESR due to the FMN free radical. Under anaerobic conditions the free radical formed on illumination is stable for at least 50 minutes in the dark. If O_2 is then introduced into the solution, in the dark, the free radical is rapidly destroyed. As long as O_2 is present, illumination does not induce free-radical formation, but when the solution is again freed of O_2, the light-induced signal is observed. These results indicate that absorption of light causes a partial reduction of FMN to a free-radical form and that the free radical may be reoxidized to FMN by molecular O_2.

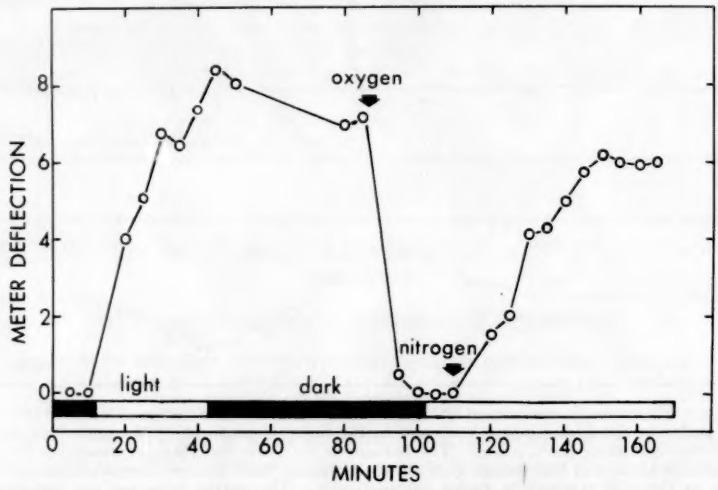

FIG. 2.—The effect of light and of oxygen and nitrogen on the ESR obtained from a 10^{-2} M solution of FMN. The maximum meter deflection is plotted against time. The solution was nitrogenated at the start of the experiment.

FMN is readily reduced by suitable reducing agents. These include H_2 and palladium black, borohydride, and reduced diphosphopyridine nucleotide (DPNH). The last substance is of special interest because it represents a hydrogen donor in a number of enzymatic systems in which FMN functions as a prosthetic group. Singer *et al.* have shown that the coupled oxidation-reduction of DPNH and FMN (or FAD or riboflavin) will occur even in the absence of an enzyme,[7] and we have observed earlier that this process is accompanied by the appearance of an ESR signal.[8]

When the ESR signals yielded by a neutral solution of FMN after illumination, after reduction by sodium borohydride, and after reduction by DPNH are compared (Fig. 3), it becomes evident that they are due to the same free radical. In all cases the ESR signal is centered about a g-value of about 2.004 and exhibits the same hy-

perfine structure. The degree to which the hyperfine peaks are resolved in the three situations differs. Resolution of hyperfine structure may be diminished by the exchange of unpaired electrons among the components of a solution.[9] Such an effect is to be expected, for example, from electron exchange between FMN and DPNH molecules. Apart from such differences in resolution, the ESR signals shown in Figure 3 appear to possess identical hyperfine structures. Since hyperfine structure is a specific function of the number and character of the atoms which interact with the free radical's unpaired electron, it appears that the three procedures form the same free radical.

That the free radical formed by light is identical with the free radical which occurs

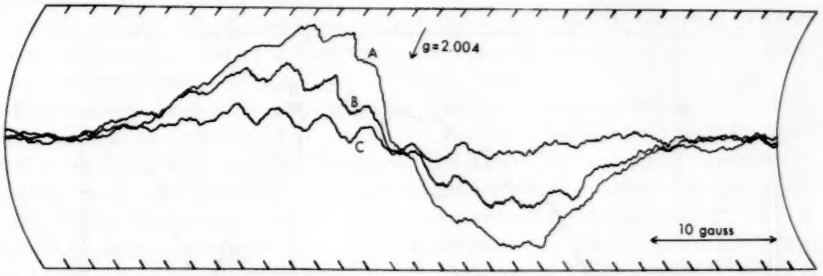

FIG. 3.—ESR of a 10^{-2} *M* solution of FMN reduced by various methods. Curve *A* represents FMN reduced with DPNH; curve *B*, an illuminated solution of FMN; curve *C*, FMN reduced with borohydride. Ordinate and abscissa are as in Fig. 1.

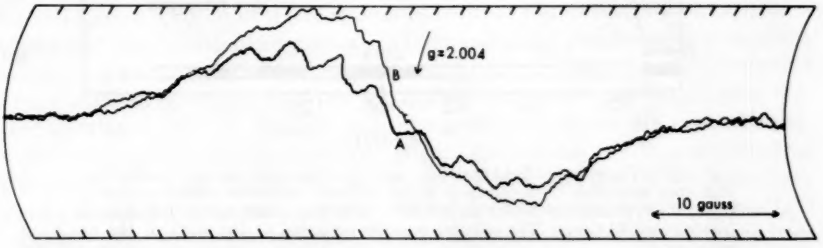

FIG. 4.—ESR obtained from a 10^{-2} *M* solution of FMN in phosphate buffer at pH 7. Curve *A* represents the resonance obtained from an illuminated solution of FMN; curve *B* represents the effect of the subsequent addition in the dark of 10 mg. of DPNH and dilution with 2 volumes of buffer. Ordinate and abscissa are as in Fig. 1.

as an intermediate in the biochemically significant reduction of FMN by DPNH is confirmed by the experiment shown in Figure 4. When DPNH is added to an illuminated solution of 10^{-2} *M* FMN, the intensity of the ESR signal increases significantly, but the *g*-value and hyperfine structure remain unchanged.

The foregoing results have been extended to oxidation-reduction enzymes which contain FMN and FAD. Results obtained from illumination of solutions of the lactic oxidative decarboxylase described by Sutton[10] are shown in Figure 5. This is a highly purified crystalline enzyme, containing FMN, which oxidizes and decarboxylates lactic acid, with molecular oxygen serving as hydrogen acceptor. Sutton

postulates that a free-radical intermediate occurs during the activity of this enzyme, and we have recently confirmed this expectation by means of the ESR technique.[11]

In the absence of lactate, the enzyme does not exhibit an ESR signal in the dark. If it is illuminated under anaerobic conditions, an ESR signal appears which is stable in the dark. Oxygenation in the dark destroys the signal, which is readily regenerated when the oxygen is again removed and the sample is illuminated. Similar results have been obtained with the old yellow enzyme which contains an FMN prosthetic group and with D-amino acid oxidase, which contains FAD. As yet the ESR signals obtained by illuminating enzyme solutions have not been sufficiently intense to permit detection of hyperfine structure. However, these signals show g-values and half-widths which resemble those of the FMN free radical already

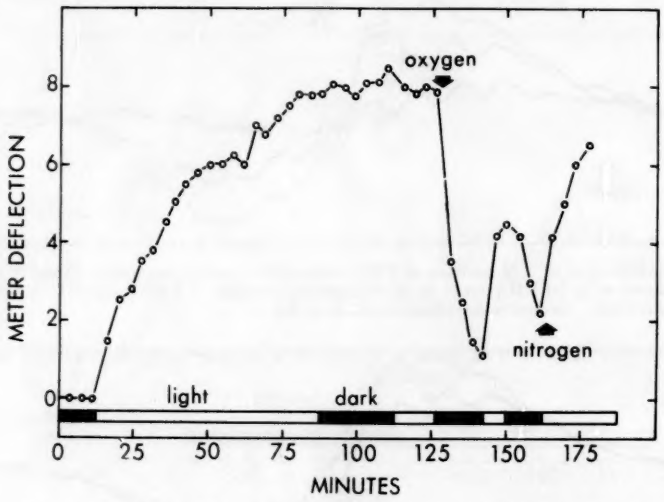

FIG. 5.—The effect of light and dark and of oxygen and nitrogen on the electron-spin resonance obtained from 8 mg. of lactic oxidative decarboxylase in 0.15 ml. of phosphate buffer at pH 5.9. The maximum meter deflection is plotted against time. The solution was nitrogenated at the start of the experiment.

described. Thus FMN either free or as the prosthetic group of an enzyme, when illuminated, forms a partially reduced free-radical intermediate.

IV. *Discussion.*—Michaelis[12] has described two types of intermediates which result from partial reduction of riboflavin. From electrometric titrations he concluded that both are free radicals. The red intermediate—rhodoflavin—is produced when reduction occurs in a strongly acid medium; the green intermediate—verdoflavin—occurs when riboflavin is reduced in neutral or alkaline conditions.

The foregoing experiments were carried out with neutral solutions of FMN; and in all cases the partially reduced solutions developed a greenish color. Therefore, the observed ESR signals can be ascribed to a free radical of the verdoflavin type. That free radicals formed by chemical reduction of riboflavin and FMN exhibit ESR's has been reported previously.[13, 14]

Preliminary experiments show that when an acid solution of FMN is illuminated, red color develops, and an ESR signal is observed. The hyperfine splitting is identical with that yielded when an acid solution of FMN is reduced by means of zinc, but the resonance is significantly different from that due to the neutral free radical. Like the latter, the acid free radical is reoxidized by molecular O_2. Thus the general effects observed with the free radical formed in neutral solutions pertain as well to the acid form of the FMN free radical, although the electronic structure of the two forms appears to be different.

There is now a significant literature regarding the effects of visible light on oxidation-reduction enzymes and on related metabolic processes. Most directly related to the foregoing work are the results of Merkel and Nickerson,[15] who found that riboflavin is reduced when illuminated in the presence of a metal-chelating agent. Polarigraphic evidence suggested that a free-radical intermediate occurred, and the transitory appearance of a red color was noted when photochemically reduced solutions were reoxidized in air.

Direct observations show that illumination causes marked changes in the activity of flavin enzyme systems. Early work by Bernheim and Dixon[2] and the later studies by Galston[3] showed that illumination enhances the activity of flavoprotein enzymes, such as xanthine oxidase and indoleacetic acid oxidase. On the other hand, White and Vernon[16] report that illumination inhibits the activity of the DPNH oxidase of *Rhodospirillum rubrum* chromoplasts. These authors find that the effect is significantly diminished by the addition of FMN, and they suggest that the inhibitory effect is probably localized at a flavin-bearing component of the electron transport system.

Most studies of the effect of light on the rate of cellular respiration have been directed toward analysis of the relationships between respiration and photosynthesis and deal largely with plant material. Illumination of nonphotosynthetic plant tissues, such as roots, increases oxygen consumption 20–100 per cent. The effect is maximal with wave lengths below 500 mμ, and is probably due to a yellow pigment such as a flavin.[17] Results with photosynthetic tissues are contradictory. In certain algae, light causes a marked stimulation of respiration; in others no effect is observed.[18] These effects may be associated with changes in the oxidation-reduction level of the various components of cellular electron transport systems.[19]

The foregoing results are consistent with the expected effects of light-induced free-radical formation on the course of enzyme processes in which flavins participate. Thermodynamic considerations, which have been described in detail by Michaelis,[12] indicate that the rate of electron transport mediated by a given enzyme will be influenced by the level of steady-state concentration of free-radical intermediates which the enzyme attains. In a simple in vitro system, exposure to light is likely to enhance the rate of electron transport and hence increase the rate of enzyme activity. However, in a sequential oxidation-reduction system a light-induced change in the free-radical level of a given constituent will in turn alter the redox balance and turnover rate of other constituents of the system. In this case one cannot predict what over-all change, if any, in the rate of electron transport by the entire system will occur. These considerations and the relative ease with which light-induced flavin free radicals are formed suggest that these substances may be frequently responsible for the foregoing effects of light on the activity of metabolic systems.

Some further consequences of these observations merit consideration. Commonly encountered intensities of light will induce detectable free-radical formation in flavin systems. For example, a solution of FMN exposed to a 150-watt lamp at a distance of 1 foot contains about 1 per cent of free radical after 3 hours; a 15-minute exposure to full sunlight yields about 0.5 per cent free radical. Being relatively stable in the absence of O_2, a flavin free radical may persist in the dark for some time after its photochemical formation. This means, for example, that in the preparation of a flavoprotein enzyme, exposure to ordinary room light may have a persistent effect on the material's free-radical content and subsequent behavior.

Finally, these results suggest a mechanism for some of the ecological effects of light. Light intensities that are capable of producing a significant steady-state level of flavin free radicals lie within the range of terrestrial illumination. The free radicals formed in this way appear to be identical with those which occur as intermediates in the oxidation-reduction of flavin prosthetic groups. Taken together, these facts lead to the expectation, which appears to be borne out by the available evidence, that ordinary daylight may play a significant role in metabolic activity. This phenomenon may in turn mediate some of the numerous effects of light on cellular processes. These observations also support an interesting suggestion, due originally to Oparin,[20] that enhancement of the efficiency of biochemical electron transport by absorption of daylight may have figured prominently in the evolution of modern forms of metabolism.

* This work was supported in part by the Office of Naval Research, by research grant C-3983 from the National Cancer Institute, Public Health Service, and by the United States Air Force through the Air Force Office of Scientific Research of the Air Research and Development Command.

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*LINEAR ANALYSIS OF TOBACCO MOSAIC VIRUS**

By Barry Commoner, Georgia B. Shearer, and Clarice Strode

HENRY SHAW SCHOOL OF BOTANY, WASHINGTON UNIVERSITY, ST. LOUIS, MISSOURI

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I. *Introduction.*—Tobacco mosaic virus (TMV) is a basically linear structure. It is a hollow cylinder 3000 Å long and 150 Å in diameter. About 2,100 protein subunits are arranged in a flat helix to form the hollow rod.[1] A single strand of nucleic acid (RNA)[2,3,4] is fully inclosed in the rod, forming a helix which lies in the interstices between adjacent turns of the helix formed by the protein subunits.[5] Thus both the virus RNA and the virus protein subunits form single linear structures arranged in coincident helixes. TMV exhibits a specific biological competence which must derive from the composition and configuration of its substance.

The present paper reports the results of experiments designed to study the effects of graded linear dissociation of the virus structure on the infectivity of TMV. Measured linear sections of virus protein and RNA are removed from one end of the virus rod. Analysis of the detached substance and of the biological competence of the residual rod lead to a description of the linear distribution of certain chemical and biological attributes of TMV.

II. *Experimental.*—This work derives from electron-micrograph observations reported by Hart.[6] These show that a brief-exposure of TMV to a heated solution of detergent (0.02 per cent Duponol) removes protein subunits from the virus progressively, beginning at one end of the rod, so that a terminal section of the virus RNA is thereby exposed. Hart finds that after a 15-second treatment at 85°, more than 90 per cent of the rods are affected at only one end.[2]

Other evidence indicates that the two ends of the TMV rod are unlike. TMV has a pronounced tendency to form dimers. However, since no higher aggregates occur, the two ends of the dimer are incapable of forming a link with another rod.[7,8] Hence one end of the virus rod is able to form an end-to-end complex, while the other end is inactive. A distinction between the ends of the virus is also inherent in the structure of RNA, which is directional. Since the single RNA fiber is tightly held between adjacent protein layers, it is likely that the ease with which protein subunits are removed will depend on the character of the associated RNA. These considerations indicate that the two ends of the TMV rod are unlike and that protein is preferentially removed from one of them by treatment with detergent.

In the experiments described below, protein is removed from preparations of the common strain of TMV by heating in solutions of 0.02 per cent Duponol for 5–20 seconds at 75° or 80° C. This treatment is less vigorous than Hart's mildest treatment (which resulted in the highest proportion of single-end stripping), so that the proportion of rods in which protein is removed from both ends is probably less than 10 per cent.

To determine the homogeneity of the product of the stripping process, a virus preparation was heated in detergent and analyzed chromatographically on a column of Ecteola according to a method described earlier.[9] Ordinary TMV is eluted from such a column with relatively dilute salt solution (0.1 *M* NaCl). In contrast, the treated nucleoprotein is not removed from the column until 0.3 *M* NaCl is used.

Analysis of the eluates shows that less than 5 per cent of the treated nucleoprotein is unaltered TMV. Thus the detergent stripping process yields a homogeneous product composed of nucleoprotein particles, nearly all of which have lost protein from one end.

The effects of the stripping process and of subsequent treatments of the virus rod may be determined quantitatively by analysis of the non-sedimentable material (the supernate following ultracentrifugation at 100,000 *g* for 45 min.) removed by the treatment and of the remaining sedimentable rod for protein and RNA content. This is readily accomplished by means of the Folin analysis (for protein) and UV spectrometry of extracts prepared in 0.5 *M* perchloric acid at 90° (for RNA).

Such analyses show that the non-sedimentable material removed from the rod by treatment in hot detergent solution is wholly protein and that the residual rod loses a corresponding amount of protein while retaining all its RNA. Since this process affects only one of the two unlike ends of the virus rods, the terminal protein-free

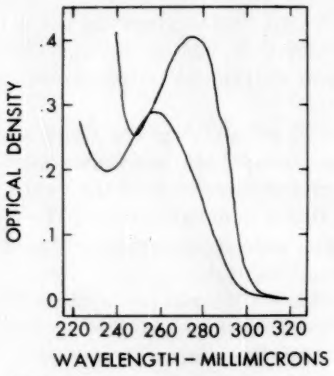

FIG. 1.—Absorption spectra of the non-sedimentable material removed from TMV rods by a 15-second treatment at 80° C. (*upper curve*) and by the subsequent treatment of the residual rods with pancreatic ribonuclease (*lower curve*).

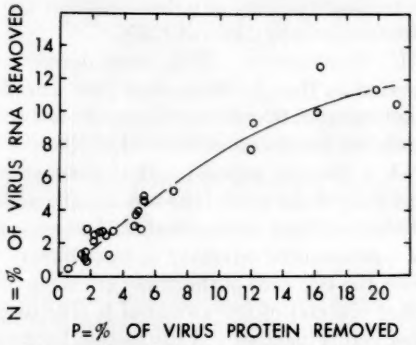

FIG. 2.—The relationship between the per cent of virus protein removed by the detergent treatment and the per cent of virus RNA removed by a subsequent treatment of the protein-stripped rod with pancreatic ribonuclease.

section comprises a fraction of the total length of the virus equal to the quotient: protein removed/protein in original TMV. Removal of protein exposes a corresponding linear section of the RNA fiber, and the same quotient is also a measure of the contour length of the exposed terminal section of the virus RNA.

By the use of pancreatic ribonuclease it is possible to remove a measurable part of the exposed section of RNA. Previous efforts to employ pancreatic ribonuclease as a means of studying the accessibility of the RNA in TMV have been hampered by the enzyme's tendency to form a firm, unifectious complex with the virus.[10] We find that the presence of 0.5 *M* NaCl in the medium during incubation with pancreatic RNAase prevents complex formation without reducing the rate of enzyme

activity. When ordinary TMV is treated with pancreatic RNAase under these conditions, no RNA is removed from the virus and no appreciable change in infectivity is observed (Table 1).

In contrast, after protein is removed from a terminal section of the virus rod by heating in detergent solution, treatment with RNAase yields non-sedimentable nucleic acid fragments (see Fig. 1). Figure 2 shows the results of a series of determinations of the amount of RNA removed by RNAase treatment of TMV rods from which a given terminal portion of the virus protein has been previously removed by the heat-detergent technique. The amount of RNA removed is a function of the length of the RNA fiber previously exposed by removal of protein. When up to about 3 per cent of the terminal protein subunits have been stripped off, subsequent treatment with RNAase removes a nearly equal fraction of RNA. When longer sections of the virus protein are removed, the enzyme treatment detaches only a part of the exposed RNA, and the portion which remains attached to the rod comprises 1–10 per cent of the total virus RNA. Internucleotide bonds that are susceptible to pancreatic ribonuclease therefore occur with great frequency in the terminal 3 per cent section and less frequently in the more interior parts of the RNA fiber.

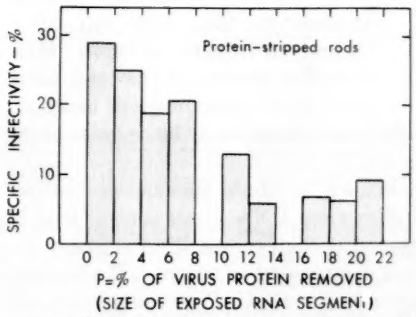

FIG. 3.—The relationship between the specific infectivity of modified TMV rods and the per cent of virus protein removed from one end of the rod (which is equal to the per cent of RNA exposed). Each bar represents the average infectivity for 1–10 samples. Specific infectivity determined by relative lesion counts of treated sample and of an equal concentration of untreated TMV on opposite halves of leaves of *Pinto* bean.

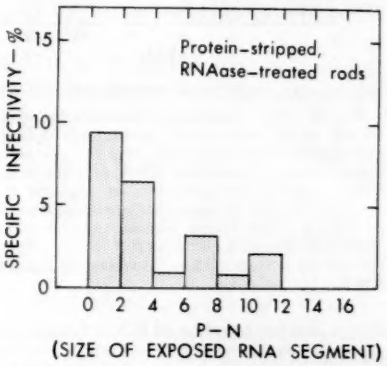

FIG. 4.—The relationship between the specific infectivity of modified TMV rods and the per cent of the virus RNA which is exposed following protein removal and treatment with pancreatic ribonuclease. P represents the per cent of virus protein removed from one end of the rods by the detergent treatment. N represents the per cent of virus RNA removed from the end of the RNA fiber by means of pancreatic ribonuclease, after the RNA has been exposed by removal of protein. The size of the section of RNA still attached to the rod, though exposed, after ribonuclease treatment is represented by $P - N$. Bars represent average specific infectivities for 1–5 samples. Specific infectivity determined as described in legend of Fig. 3.

When protein alone is removed from a terminal portion of the virus rod, the infectivity of the rod is reduced, the effect being a function of the length of the affected section. Figure 3 shows that the removal of a terminal 2 per cent section of protein or less, reduces specific infectivity to an average of about 30 per cent and that the level of infectivity is further reduced as progressively longer sections of protein are removed.

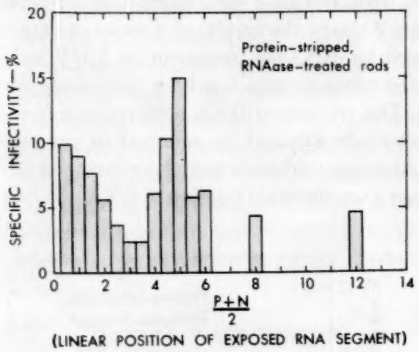

FIG. 5.—The relationship between the specific infectivity of rods which, after both detergent and ribonuclease treatment, retain 2 per cent or less of exposed, attached RNA, and the original linear position occupied by the exposed RNA section. P and N have the meanings described in Fig. 4. The linear position of the exposed section in the rod is equal to $(P + N)/2$. Specific infectivity determined as described in legend of Fig 3.

If rods containing intact RNA, partially exposed by the previous removal of a terminal section of protein, are then treated with RNAase, significant changes in infectivity occur. The effects are described in Figures 4 and 5. Figure 4 shows that the removal of RNA, in general, reduces the level of infectivity and that (as in the case of protein-stripped rods) the infectivity is inversely related to the length of the extruding section of RNA fiber. When the exposed section of RNA that remains attached to the virus rod after RNAase treatment is more than about 3–4 per cent of the total virus RNA fiber, infectivity is minimal. When shorter lengths of RNA remain attached, infectivities may be as great as 20 per cent of that of TMV.

Figure 5 shows that the infectivity exhibited by those ribonuclease-treated rods which retain relatively short lengths of exposed RNA (2 per cent or less) is also a function of the original linear position that the exposed RNA segment occupies in the virus rod. The linear position is estimated as follows: If the heat-detergent treatment removes a terminal protein section that is P per cent of the total virus protein, and subsequent RNAase treatment removes a terminal section of the exposed RNA that is N per cent of the total virus RNA, then the center of the section of RNA that remains exposed but attached to the rod lies at the distance $(P + N)/2$ from the affected end of the rod.

When the exposed section lies within 0.5 per cent of the affected end of the virus, the average specific infectivity is about 12 per cent. With increasing distance from the end, the infectivity declines, reaching a minimum of about 2 per cent specific infectivity when the exposed RNA section is located at about 3 per cent from the end of the virus. When the exposed section occupies a slightly more interior position, the specific infectivity exhibits a surprising increase, attaining a peak of about 20 per cent at a position 5 per cent from the end of the rod. Beyond this point the infectivity declines again, so that rods with exposed sections that lie more than 5.5–6 per cent from the affected end are relatively uninfectious.

III. *Discussion.*—The foregoing results provide evidence regarding the site of the generative competence of the virus. In the ordinary intact virus rod, RNA is

wholly inaccessible to attack by pancreatic ribonuclease; the terminal sections of the protein subunit helix are therefore free of overlying RNA and themselves effectively cover the underlying RNA. Such rods are, of course, maximally infectious, but there is evidence that the infection process starts only some hours after the virus enters the host, and after the virus RNA becomes exposed.[11] Intracellular exposure of virus RNA is most probably accomplished by the removal of the terminal layer of protein. This suggests that the infectivity of TMV is due to an exposed section of RNA which is accessible, though still bound to the underlying protein subunits.

Results of experiments with TMV modified by the stripping technique support this view. Rods freed of a short terminal section of protein are the experimental analogues of the natural virus in the phase following removal by host processes of the terminal protein layer. Such rods exhibit a considerable infectivity which is frequently equal to that of ordinary TMV. But if the exposed section of RNA is then removed by RNAase, the infectivity is reduced.

The diameter of the cylindrical zone occupied by the virus RNA is about 40 A,[1] so that the contour length of a single turn of the RNA helix is about 250 A, or about

TABLE 1

THE EFFECT OF PANCREATIC RIBONUCLEASE ON INTACT TMV

TMV Preparation	Non-sedimentable Nucleic Acid Due to Ribonuclease Treatment (Per Cent of Total Virus RNA)	Specific Infectivity*	
		Control† TMV	Ribonuclease treated TMV‡
1	0.14	69	74
2	0.09	57	54
3	0	56	58

* Specific infectivity determined from the ratio of the number of lesions produced by the sample to the number of lesions produced by an equal amount of untreated TMV, inoculated on opposite half-leaves of *Pinto* bean.
† As control, TMV was incubated in pH 7 phosphate buffer for 1 hour at 25°.
‡ Incubated in 1 μg. of crystalline pancreatic ribonuclease per ml. of pH 7 phosphate buffer for 1 hour at 25°.

0.8 per cent of the total length of virus RNA. Hence, if an exposed RNA section has a length of about 1 per cent, it can be wholly accommodated in its normal position bound to the surface of the underlying protein subunits. If a greater length of the RNA fiber is exposed, the distal portion will project free.

These considerations suggest an explanation for the reduced infectivity observed when the exposed section of RNA attached to the end of a modified virus rod is greater than 3–4 per cent of the total RNA. Being a single strand, the virus RNA cannot by itself maintain a secondary structure. Since a specific configuration is probably essential to any replicative process, it would appear that only that part of the exposed RNA which is held in a specific configuration through association with protein subunits is biologically active (i.e., about 1 per cent). Additional distal RNA will tend to collapse in a random position over the highly ordered proximal portion. This may account for the inhibitory effect of RNA sections greater than about 3 per cent.

Our results show that not all portions of the virus RNA fiber are equally effective, even when present in a suitably attached form. The section of virus RNA that originally occupies a position 4–6 per cent from the affected end of the rod is unusually efficient in infectivity. This may be due to (*a*) the presence of an especially suitable nucleotide structure in this region of the RNA fiber, (*b*) the presence at this

position in the rod of protein subunits which are unusually effective in imposing on the attached RNA the configuration necessary for infectivity, or (*c*) a combination of both these effects.

The stripping technique also provides opportunities for investigating possible variation in the composition of virus RNA and protein subunits at successive linear positions in the rod. By using sufficient starting material, it is possible, for example, to remove a given terminal section of the virus RNA for analysis with respect to nucleotide ratios. Preliminary studies of this type show that when the section analyzed is sufficiently small, distinctive deviations from the over-all nucleotide ratios are observed.[12]

These results show that the technique of linear decomposition of TMV yields useful data regarding the relations between the biological activity and structure of the virus. The present data suggest that the biological competence of TMV is associated with approximately a single turn of the helix formed by the virus RNA, provided that this section of the RNA fiber is bound to the surface of the underlying protein subunits.

* Aided by a grant from the National Foundation for Infantile Paralysis and the Rockefeller Foundation.

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A SYNTHESIS OF RIBOTHYMIDINE-5′-PYROPHOSPHATE AND ITS CONVERSION TO POLYRIBOTHYMIDYLIC ACID

By Beverly E. Griffin, Sir Alexander Todd, and Alexander Rich

UNIVERSITY CHEMICAL LABORATORY, CAMBRIDGE, ENGLAND, AND DEPARTMENT OF BIOLOGY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY, CAMBRIDGE

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Introduction

One of the more interesting developments in polynucleotide chemistry has been the isolation of an enzyme which, acting on ribonucleoside-5′-pyrophosphates, yields inorganic phosphate and polyribonucleotides. In its reverse reaction the enzyme breaks down the polynucleotide chain by phosphorolysis, and it has therefore been described as polynucleotide phosphorylase by its discoverers, Grünberg-Manago and Ochoa.[1] It has been shown both chemically and enzymatically that the synthetic polynucleotides prepared by means of polynucleotide phosphorylase have the same recurring 3′:5′-internucleotidic linkage as the naturally occurring ribonucleic acids,[2] and, as a result, much work has been devoted to exploring the possibility that the enzyme may play a part in the synthesis of polyribonucleotides in vivo. Another consequence of the discovery of polynucleotide phosphorylase is, however, that it enables us to produce virtually at will a variety of polynucleotides with controlled compositions and with different chain lengths. Such products provide valuable materials for studying the influence of composition and molecular size on such properties as acid-base titration, molecular configuration, and enzymatic specificity and thereby developing further our knowledge of nucleic acid chemistry.

The synthetic polynucleotides exhibit a number of properties which depend on the specificity of purine-pyrimidine interactions. Thus it was early found that polyadenylic acid and polyuridylic acid will wrap around each other in solution to form a two-stranded helical molecule in which the adenine and uracil residues are held together by hydrogen-bonding in a manner analogous to that postulated for adenine and thymine in deoxyribonucleic acids.[3]

More recently it has been shown that this two-stranded complex can take up another chain of polyuridylic acid and become three-stranded.[4] These experiments, together with others involving polyinosinic acid,[5] have clearly demonstrated the importance and the specificity of purine-pyrimidine interactions in the formation of helical polynucleotide complexes. In order to test various theories concerning the significance of these interactions, it would clearly be desirable to prepare a number of synthetic polynucleotides containing a variety of different purine and pyrimidine bases. Hitherto, only five substrates have been found which yield polynucleotides with polynucleotide phosphorylase—the 5′-pyrophosphates of the nucleosides, adenosine, guanosine, uridine, and cytidine, which occur naturally in ribonucleic acids, and inosine-5′-pyrophosphate. The fact that the same enzyme (or enzyme system) is able to produce polynucleotides from these five substrates suggested that it may not have a high specificity relative to the purine or pyrimidine base present. This is in some degree borne out by the present work. 3-β-D-

Ribofuranosylthymine[6] (conveniently described as ribothymidine) was converted to its 2′:3′-*iso*propylidene derivative by condensation with acetone in the presence of toluene-*p*-sulphonic acid. From this substance ribothymidine-5′-pyrophosphate was synthesized by a method analogous to that already described for thymidine-5′-pyrophosphate.[7] Using polynucleotide phosphorylase, the synthetic pyrophosphate was converted to polyribothymidylic acid.

EXPERIMENTAL

3-β-D-Ribofuranosylthymine (ribothymidine).—The nucleoside was synthesized by a slight modification of the method described by Fox *et al.*[6] It was found that the yield of the intermediate tri-O-benzoyl-β-D-ribofuranosylthymine was increased to 60 per cent (lit. 50 per cent) by using 2 moles of 2:3:5-tri-O-benzoyl-D-ribosyl chloride and 1.5 moles dithyminyl-mercury and heating the mixture in xylene for 2 hours.

2′:3′-Isopropylidene-ribothymidine.—The above nucleoside (1 gm. ≡ 1 mol.) was added to a solution of toluene-*p*-sulphonic acid (7.38 gm. ≡ 10 mol.) in dry acetone (150 cc.), and the resulting solution was stirred magnetically for $1^1/_2$ hours at room temperature before being poured into a slight excess of aqueous sodium bicarbonate (170 ml. of 0.5 *N*). The mixture so obtained was evaporated to dryness in vacuo, the residue dissolved in water (*ca.* 40 ml.), and the solution extracted continuously with ethyl acetate for 6 hours. The ethyl acetate extract (*ca.* 25 ml.) was evaporated to dryness, and the resulting glass was stirred with fresh ethyl acetate (15 ml.). The mixture was filtered, the filter residue washed with a little ethyl acetate, and the combined filtrate and washings evaporated. The product, a colorless amorphous powder (1.09 gm.), was virtually pure; on Whatman No. 1 paper, using the solvent system *n*-butanol/water (86/16), it ran as a single ultraviolet-absorbing spot R_f, 0.75 (ribothymidine itself had R_F, 0.30 in this system). The iso*propylidene derivative* was crystallized by dissolving in ethyl acetate (7 ml.), adding light petroleum (b.p. 40°–60°) to incipient turbidity, and setting the mixture aside. Recrystallized from this mixture of solvents, it was apparently still hydrated, since on heating it shrank at 75° and melted at 112° (found in material dried in vacuo over phosphoric oxide for 24 hours: C, 52.0; H, 6.2; N, 9.2. $C_{13}H_{18}O_6N_2$ requires C, 52.3; H, 6.1: N, 9.4 per cent).

Ribothymidine-5′-Pyrophosphate.—A solution of 2′:3′-*iso*propylidene-ribothymidine (1 mol.) was treated with O-benzylphosphorous OO-diphenylphosphoric anhydride (2 mols.), as described by Kenner, Todd, and Weymouth,[8] and the crude 2′:3′-*iso*propylidene-ribothymidine-5′-benzyl phosphite was purified by precipitation from chloroform solution with *n*-heptane; it was obtained as a thick yellowish oil (yield *ca.* 90 per cent), which, on paper chromatography on Whatman No. 1 paper using *n*-butanol/water (86/14), showed a single ultraviolet-absorbing spot of R_f, 0.84.

A mixture of the above phosphite (2.415 gm. ≡ 1 mol.) and N-chlorosuccinimide (0.715 gm. ≡ 1 mol.) in a mixture of dry benzene (60 ml.) and methyl cyanide (6 ml.) was set aside at room temperature for 2 hours, moisture being excluded. Monobenzyl phosphate (1.01 gm. ≡ 1 mol.) and triethylamine (1.48 ml. ≡ 2 mol.) were added, the mixture stirred for 2 hours, then filtered, the residue of

triethylammonium chloride being washed with benzene (10 ml.). The combined filtrate and washings were evaporated in vacuo, yielding a thick oil which was dissolved in water (50 ml.). To the filtered aqueous solution, ethanol (50 ml.) was added, together with a little acetic acid (4 drops) to give a pH of 3–4, and debenzylation was effected by hydrogenating at atmospheric pressure during 6 hours, using a palladium black catalyst (0.2 gm.). Catalyst was removed by filtration, and the solution set aside for 3 days to insure removal of the *iso*propylidene residue. Examination of the product at this stage by paper chromatography in the system *n*-butanol/acetic acid/water (5/2/3) showed it to contain one major constituent (R_f 0.14) together with three minor faster-moving components containing phosphorus and showing ultraviolet absorption, as well as some inorganic phosphate and another material which may have been ribose phosphate, since some thymine could be detected in the solution by electrophoresis.

The solution was concentrated in vacuo to remove ethanol, brought to pH 6 with lithium hydroxide, and a saturated solution of barium acetate (2.55 gm.) was added. Ethanol (5 vol.) was now added, and the mixture left overnight. The precipitate was crude *barium ribothymidine-5′-pyrophosphate* (3.21 gm. corresponding to 92 per cent of theory, assuming the salt to be a tetrahydrate); this material contained only one ultraviolet-absorbing substance but was contaminated with a small amount of barium phosphate. Pure barium ribothymidine-5′-pyrophosphate was prepared from this product by dissolving in hydrobromic acid (0.1 *N*), adding barium hydroxide (0.3 *N*) to pH 5, and precipitating with ethanol (5 vol.). After four such precipitations, the salt was entirely free of inorganic phosphate (found in air-dried material: C, 17.4; H, 3.1; N, 4.2. $C_{10}H_{13}O_{12}N_2P_2{}^3/_2Ba{\cdot}4H_2O$ requires C, 17.3; H, 3.05; N, 4.0 per cent).

Polyribothymidylic Acid.—The barium salt of ribothymidine-5′-pyrophosphate was converted to the sodium salt by passage through the sodium form of a Dowex 50 ion exchange column. For the polymerization, two different enzyme preparations were used. One of these was from *Azotobacter vinelandii* and was kindly supplied by Professor S. Ochoa. The other enzyme preparation was made from *Escherichia coli*, using the method of Littauer and Kornberg,[9] as described previously.[10] The polymerization was carried out using a reaction mixture containing, in each milliliter, 45 μ moles of the pyrophosphate, 10 μ moles of $MgCl_2$, 150 μ-moles of tris buffer, and 0.005 ml. of the enzyme preparation. With the *Azotobacter* enzyme, the buffer was adjusted to pH 8.1, while for the *E. coli* enzyme, the pH was 7.4. The incubations were carried out at 37° C. The activity of the enzyme preparations was checked by running a parallel control, using one of the natural nucleoside pyrophosphates. The results of one such experiment are shown graphically in Figure 1; the inorganic phosphate released is plotted as a function of time for the preparation of polyribothymidylic acid (poly T), as well as polycytidylic acid (poly C). It can be seen that both pyrophosphates stimulated the release of inorganic phosphate in a more or less parallel manner, reaching a plateau in approximately 6 hours. In this experiment, the yield of polyribothymidylic acid was just under 30 per cent, while that of polycytidylic acid was just over 50 per cent. In other parallel experiments comparing the synthesis of polyuridylic acid with polyribothymidylic acid, the thymine derivative was again only about half as effective as measured by the percentage yield at equilibrium. However,

it should be noted that ribothymidine pyrophosphate is much more active in producing a polynucleotide than is guanosine pyrophosphate. As the enzyme preparations used had not been exhaustively purified, no lag period was observed in any of the reactions studied.

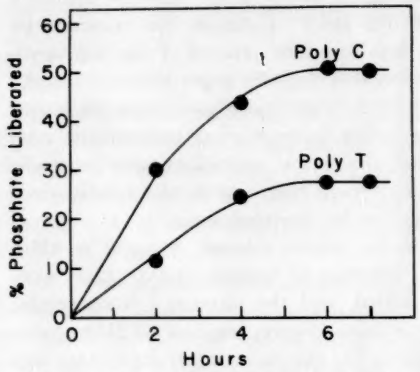

FIG. 1.—Release of inorganic phosphate during the formation of polyribothymidylic acid (poly T) and polycytidylic acid (poly C).

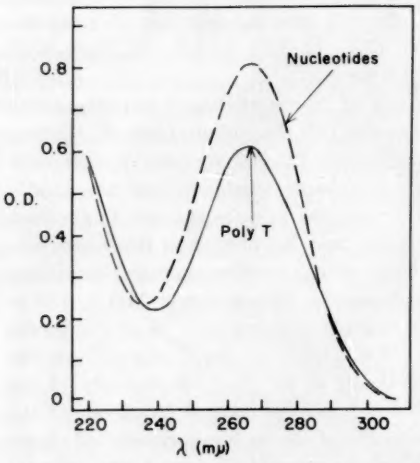

FIG. 2.—Ultraviolet absorption spectrum of polyribothymidylic acid (poly T) in 0.1 *M* NaCl, 0.01 *M* sodium cacodylate buffered to pH 6.8. The broken line represents the absorption spectrum after the polymer had been hydrolyzed in alkali and then returned to neutral pH.

After equilibrium was reached, the polymer was precipitated by the addition of 2 volumes of cold ethanol. The precipitate was redissolved in distilled water, and then dialyzed in the cold against 5×10^{-3} *M* NaCl. The polymer solution was then dried in the frozen state to produce a white fibrous material, which had the same appearance as the other synthetic polynucleotides.[2]

The ultraviolet spectrum of polyribothymidylic acid is illustrated in Figure 2. It has a maximum at 266 mμ, which is very close to that observed for thymine and thymidylic acid. The extinction coefficient per mole of phosphate at 266 mμ is 6.8×10^3 in 0.1 *M* NaCl, buffered to pH 6.8 in 0.01 *M* sodium cacodylate. This was measured both by carrying out phosphate analyses[11] and by hydrolyzing the polymer with alkali to a mixture of the 2′ and 3′ nucleotides. In Figure 2 the broken line represents the spectrum of the product obtained by alkaline hydrolysis (24 hours in 0.3 *N* KOH at room temperature). As is the case with most of the other synthetic polynucleotides[12] and with the naturally occurring nucleic acids, the polymer has a lower optical density than the constituent nucleotides. It should be noted that the optical density of the polymer varies somewhat with molecular weight. The figure quoted above is for the larger of the two preparations described below. Variations of as much as 6 per cent in the extinction coefficient of different preparations of polyadenylic acid have been reported.[12]

Further evidence for the structure of the polymer is provided by its quantitative hydrolysis by spleen phosphodiesterase[13] and pancreatic ribonuclease to a single

mononucleotide (presumably ribothymidine-3′-phosphate). With the latter enzyme the nucleoside cyclic phosphate and the dinucleotide with a cyclic terminal phosphate group were obtained as intermediates. We are indebted to Dr. L. A. Heppel for carrying out these experiments, which are also of considerable interest in connection with the specificity of the enzymes employed.

The macromolecular character of the polymers of ribothymidylic acid was shown by ultracentrifugal studies. Sedimentation constants were measured in a Spinco ultracentrifuge with ultraviolet absorption optics using solutions with a concentration of 20 μg/ml (i.e., sufficiently dilute to give results independent of concentration). Polyribothymidylic acid obtained using the enzyme from *A. vinelandii* has an $S_{20} = 10.9$ in 0.1 *M* NaCl, 0.01 *M* sodium cacodylate at pH 6.8. The enzyme isolated from *E. coli* was not so highly purified, and this was reflected in a smaller sedimentation constant ($S_{20} = 4.1$) of the polymer which it produced. The difference in chain length is undoubtedly associated with nuclease contaminants in the less purified sample of polynucleotide phosphorylase.

Dr. L. A. Heppel, to whom we are greatly indebted, has examined the action on ribothymidine-5′-pyrophosphate of a highly purified preparation of polynucleotide phosphorylase supplied by Professor S. Ochoa.[14] In agreement with his earlier work on adenosine and uridine pyrophosphates,[15] using this preparation polymerization occurred only after a prolonged lag period. The lag was overcome by adding the trinucleotide ($_pA\,_pA\,_pA$) as a primer; the triadenylic acid primer was incorporated into the polynucleotide produced, and early in the reaction intermediates were observed which corresponded to the addition of one, two, and three ribothymidylic acid units to the primer.

DISCUSSION

When the research described in this paper was initiated, the synthesis of a polyribothymidylic acid seemed of particular interest, since the behavior of polyribonucleotides containing the "unnatural" base thymine might help to throw some light on the physical differences between the natural ribonucleic acids and the deoxyribonucleic acids in which thymine is of universal occurrence. This possibility still warrants further study, but it seems that thymine can no longer be properly described as an "unnatural" component of polyribonucleotides, for Littlefield and Dunn[16] have recently reported the occurrence of thymine in hydrolyzates of ribonucleic acids obtained from four different micro-organisms. The amount of thymine detected corresponded to 0.6–1.0 moles per 100 moles uracil or about 1 thymine residue per 500 base residues present; on this basis it is a minor, but not necessarily a negligible, constituent of the ribonucleic acid samples examined.

Regarding the specificity of polynucleotide phosphorylase, it is interesting to note that in preliminary studies the enzyme showed no tendency to produce a polynucleotide from 5-bromouridine-5′-pyrophosphate.[2] Since the van der Waals radius of bromine is very similar to that of a methyl group (2.0 A), it would seem that the failure of the bromo-derivative to react cannot be explained on steric grounds. Further studies with this substrate would be desirable to establish whether it shows any inhibitory action on the enzyme system.

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*PARAMAGNETIC RESONANCE STUDY OF IRRADIATION DAMAGE IN CRYSTALLINE CARBOHYDRATES**

BY DUDLEY WILLIAMS, J. E. GEUSIC, M. L. WOLFROM, AND LEO J. MCCABE

DEPARTMENT OF PHYSICS, AND DEPARTMENT OF CHEMISTRY, THE OHIO STATE UNIVERSITY, COLUMBUS, OHIO

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INTRODUCTION

By subjecting organic molecules in solids to strongly ionizing radiation, such as X-rays or cathode rays, electrons can be removed from ground-state molecular orbitals with sufficient energy to free them from the molecule. When the organic molecule loses an electron in this manner, the ionized molecule, if it holds together, will have an unpaired electron in one of its orbitals. The electron which is removed from one molecule may become attached to a neighboring molcule and go into an excited orbital of this molecule, or it may be trapped at imperfections in the crystal lattice. It is possible for the ionizing radiation to produce either positively or negatively charged ions. These ions will be short-lived and will probably become stabilized as uncharged free radicals with unpaired electrons. In most cases the formation of the final radical will be a complicated process, in which the ionizing radiation will produce unstable entities, which in turn will decay to others until a stable radical is formed. If the barrier to the return passage of the electrons between the molecules is large, then sufficiently high concentrations of free radicals can be built up to give a detectable electron-spin resonance.

If the spin of the unpaired electron in the radical is entirely free from the perturbing influence of its environment, a single sharp line with $g = 2.0023$ might be expected. However, the electron is very sensitive to its environment, and information about the interaction of the electron with its surroundings can usually be obtained from the paramagnetic resonance spectrum. In the resonance spectrum

there are usually two sources of information: most importantly, the hyperfine structure, which arises from the interaction of the magnetic moment of the electron with the magnetic moments of the nuclei on which the electron is localized, and, second, the small residual spin-orbit coupling which in some cases causes the g-factor to be slightly anisotropic and different from the free-spin value.

Hyperfine Structure.—The hyperfine-structure splittings in polyatomic free radicals have been discussed in detail by Weissman.[1] If the interaction of the electron with the external magnetic field is much larger than the interaction of the electron with nuclei in the radical, the magnetic fields at which the hyperfine lines occur for constant frequency are given by

$$H = \left(\frac{h\nu}{g\beta}\right) + \frac{1}{g\beta}\sum_i A_i m_i \tag{1}$$

where A_i is the coupling constant of the electron with a particular nucleus i with spin I_i and the magnetic quantum numbers have the values $m_i = I_i, I_i - 1, \ldots, -I_i$.

If all the coupling nuclei in a given free radical have the same coupling A_i to the electron spin, one can define

$$T = \sum_i I_i \tag{2}$$

and a total magnetic quantum number $M = T, T - 1, \ldots, -T$. For this situation, equation (1) can be written in the simpler form

$$H = \left(\frac{h\nu}{g\beta}\right) + \left(\frac{A}{g\beta}\right) M. \tag{3}$$

In this case the spectrum consists of $(2T + 1)$ components separated by $\Delta H = (A/g\beta)$. The intensities of the components are proportional to the number of different combinations of m_i's which give the same value of M.

The interaction constant, A_i, of the electron spin with the moment of a particular nucleus i, in general, contains both an isotropic component and an anisotropic component. The isotropic component, which is the Fermi term,[2] is independent of the orientation of the magnetic field and arises from the non-vanishing of the electronic wave function at the nucleus in question. Since only the s atomic orbitals are non-vanishing at the nucleus, the presence of an isotropic coupling term for a particular atom in a molecule generally indicates the s character in the bonding orbitals of the atom. The anisotropic contribution of A_i comes from the direct magnetic dipole-dipole coupling of the electron with the nucleus i. In a single crystal, one can distinguish between the isotropic and anisotropic contributions to A_i by studying the hyperfine separations as the crystal is rotated with respect to the external magnetic field. For powder samples, one cannot, in general, distinguish between the isotropic and the anisotropic terms. Instead, an average A_i is obtained, because in a powder the chemical-bond axes are randomly oriented with respect to the magnetic field.

g-Anisotropy.—When the unpaired electron is localized on a non-s orbital of a single atom of a radical, the effects of spin-orbit interaction cannot be neglected. The orbital angular momentum is oriented by the strong electrical forces of the

chemical bond, and this gives rise to an anisotropy in the g-factor. If the electron-wave function is symmetric about a chemical bond, the g-factor reflects this symmetry. For an arbitrary orientation, θ, of the bond axis with respect to the external magnetic field, the observed g-factor is given by

$$g^2 = g_{||}^2 \cos^2 \theta + g_{\perp}^2 \sin^2 \theta. \quad (4)$$

In the case of a powder sample, the resonance absorption is averaged over all possible orientations of the bond axis with respect to the magnetic field. Since the $g_{\perp}$ value has greater weight than $g_{||}$, the resonance has an asymmetric shape with two peaks. The peak with maximum intensity corresponds to the $g_{\perp}$ value, and the other peak corresponds to $g_{||}$.

In a powder sample one can distinguish between hyperfine-structure effects and effects due to g-anisotropy by studying the spectrum at several different frequencies. Once the strong-field or Paschen-Back case is realized, the hyperfine-structure separation is independent of the frequency of observation. The effect due to g-anisotropy depends on the frequency of observation.

The electron paramagnetic resonance study of radiation damage to organic molecules has been pursued by a large number of workers. Gordy and co-workers[3–8] have carried out paramagnetic resonance studies of free radicals produced by X-irradiation of amino acids, peptides, fatty acids, nucleic acids, proteins, enzymes, hormones, and vitamins. Paramagnetic resonance studies on irradiated carbohydrates were reported by Combrisson and Ubersfeld[9] and by O'Meara and Shaw.[10] The results of similar studies on X-irradiated and cathode-ray–irradiated carbohydrates are reported in the present paper.

EXPERIMENTAL RESULTS

A total of sixteen irradiated carbohydrates have been studied by observing the paramagnetic resonance spectra of powder samples of these materials. α-D-Glucopyranose monohydrate, D-glucitol (sorbitol), α-D-galactopyranose, and *myo*-inositol have been studied after irradiation with both X-rays and cathode rays. In each of these samples the paramagnetic resonance spectrum of the X-irradiated sample was identical with the spectrum of the cathode-ray–irradiated sample. This result seems to indicate that the final radical products produced by X-irradiation are the same as those produced by high-energy cathode rays.

The spectrograph employed in the present study consisted of an X-band Klystron operating at constant frequency, controlled by a secondary frequency standard, which was monitored by WWV. Samples were mounted in a transmission-type resonant cavity having an unloaded Q of approximately 5000. The cavity was situated between 6-inch diameter poles of an electro-magnet supplying a strong magnetic field, which could be varied slowly by means of a clock drive attached to a potentiometer in the magnet power supply. Magnetic field modulation at 800 cycles/sec was provided by means of two small coils mounted against the external walls of the sample cavity. A lock-in amplifier was employed, so that the trace displayed on the recorded chart was a first derivative of the absorption signal.

The high-energy electron beam (cathode ray) was supplied by a resonant transformer in conjunction with a permanently evacuated cathode-ray tube. The source utilized was a 1-Mev peak, 500 μa beam-out unit.[11] The dose was measured

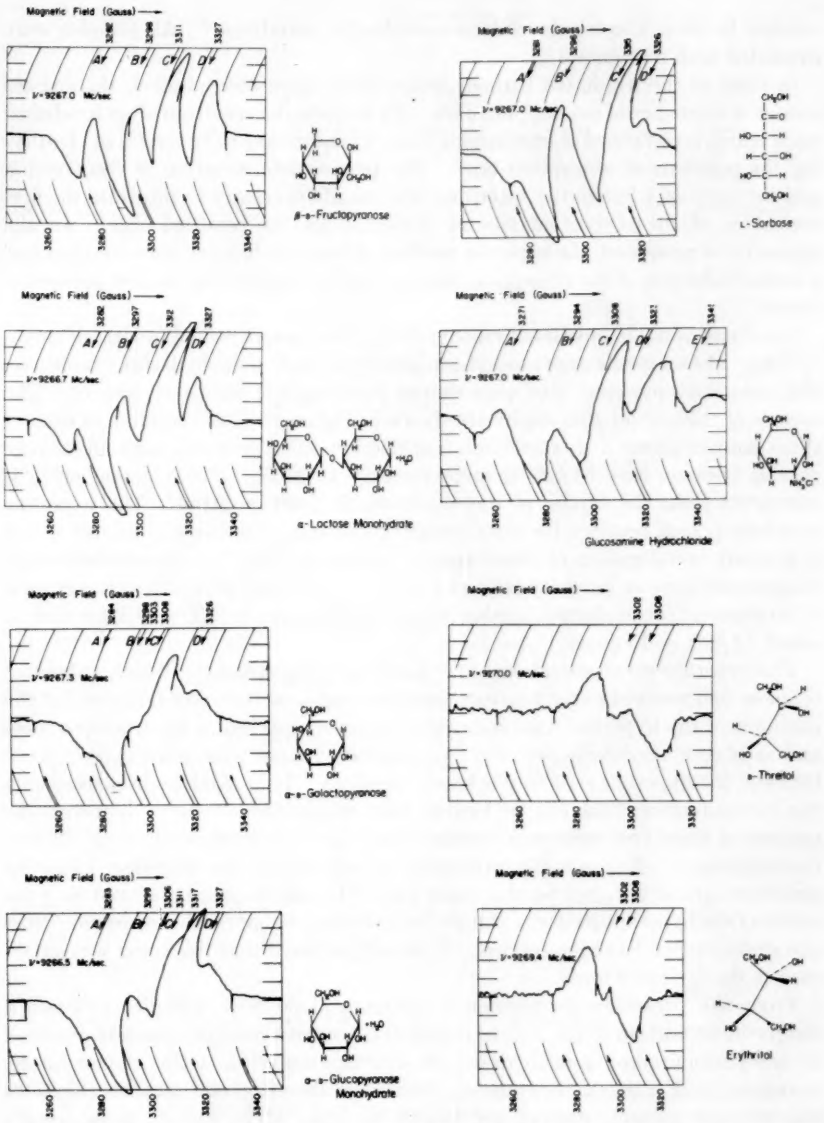

FIG. 1.—Electron-spin resonance spectra of irradiated carbohydrates. The curves represent first derivatives of the actual absorption lines.

by a specially constructed, air ionization chamber. The unit was located at the General Electric Co., Milwaukee, Wisconsin. The soft X-rays were provided by a prototype unit located at the Battelle Memorial Institute, Columbus, Ohio. The X-ray tube was operated at 60 kvp. and 200 ma. and was fitted with a beryllium

window to allow the escape of long-wave-length radiations.[12] All samples were irradiated with 5 megareps.

In most of the irradiated carbohydrates which have been studied, the spectra consist of a number of overlapping lines. To analyze the spectrum of an irradiated sugar which is composed of overlapping lines, it is necessary to be careful in identifying the positions of absorption lines. For precise determination of the position and intensity of a line in the spectrum, it is usually necessary to integrate the first derivative of the absorption spectra presented on the recorded chart. In the figures to be presented, the apparent position of lines are located, since maxima and points of inflection of the absorption curve are easily identified on the first derivative traces.

Irradiated α-D-glucopyranose monohydrate, β-D-fructopyranose, α-lactose monohydrate (4-O-β-D-galactopyranosyl-α-D-glucopyranose monohydrate), L-sorbose, and α-D-galactopyranose give very similar paramagnetic resonance spectra. The spectra of these irradiated sugars are shown in Figure 1. The spectrum of each of these samples shows a characteristic four-line hyperfine structure with an average spacing between lines ($A/g\beta$) of approximately 15 gauss. The g-factor, which is calculated from the center of the spectrum, is 2.003 ± 0.001. The hyperfine structure is best resolved for β-D-fructopyranose and α-lactose monohydrate, and a detailed investigation of these spectra indicates that the hyperfine-structure components have an intensity ratio of 1:3:3:1. This type of hyperfine structure is to be expected if the electron is interacting with three protons ($I = {}^1/_2$), for each of which ($A/g\beta$) = 15 gauss.

The hyperfine structure observed for L-sorbose (ring structure, if any, unknown) is not so well resolved as for β-D-fructopyranose and α-lactose monohydrate, but the positions of the hyperfine lines coincide with those observed in β-D-fructopyranose and α-lactose monohydrate. For α-D-galactopyranose and α-D-glucose monohydrate, the hyperfine structure is barely resolved. In α-D-galactopyranose, only the two outermost lines can be located with reasonable accuracy; however, the position of these two outermost lines is almost the same as those observed for β-D-fructopyranose. For α-D-glucopyranose monohydrate the four-line hyperfine structure is observed, but for this sugar two additional lines are observed near the center of the hyperfine pattern; this probably indicates that for this sugar more than one stable radical has been formed. It should be noted that this sugar was irradiated in the hydrated form.

From the paramagnetic resonance spectrum alone it is difficult to obtain a unique identification of the radical responsible for the hyperfine structure observed in α-D-glucopyranose monohydrate, β-D-fructopyranose, α-lactose monohydrate, L-sorbose, and α-D-galactopyranose. However, the magnetic resonance spectra indicate that possible radicals are limited to types which contain three equally coupling protons.

The paramagnetic resonance spectra observed for irradiated erythritol and D-threitol are shown in Figure 1, and those of α-L-rhamnopyranose monohydrate and *myo*-inositol are shown in Figure 2. For erythritol and D-threitol the spectrum consists of two lines of equal intensity. This type of spectrum would be expected if the electron were interacting with a single proton in the radical. The hyperfine spacing ($A/g\beta$) for erythritol and D-threitol is approximately 6 gauss. The hyper-

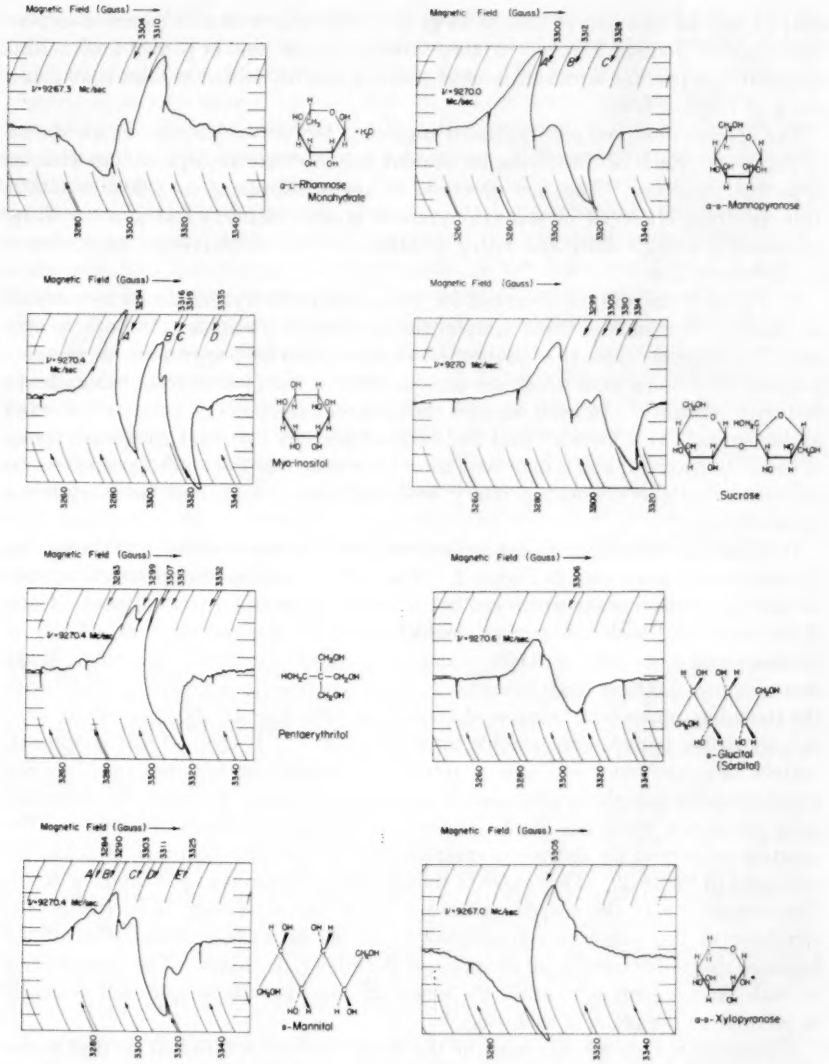

FIG. 2.—Electron-spin resonance spectra of irradiated carbohydrates. The curves represent first derivatives of the actual absorption lines.

fine structure is centered on $g = 2.003 \pm 0.001$. Two lines of equal intensity are also observed in the α-L-rhamnopyranose monohydrate spectrum; these two lines are assumed to be a hyperfine doublet with $(A/g\beta) = 6$ gauss and $g = 2.002 \pm 0.001$. In the *myo*-inositol spectrum (Fig. 2) four lines are observed. These four lines consist of a pair of intense lines and two weak lines. If the two strong lines in the spectrum, labeled A and C in Figure 2, are associated with a hyperfine doublet,

then $(A/g\beta)$ for this pair of lines is 25 gauss. The center of this assumed hyperfine-structure doublet is at $g = 2.002 \pm 0.001$. If the weaker pair of lines is also considered a hyperfine-structure doublet, then $(A/g\beta)$ for this set of lines is 19 gauss and $g = 1.991 \pm 0.001$.

The spectra observed for irradiated D-glucitol and α-D-xylopyranose are shown in Figure 2. Each of these samples showed only a single absorption line with no hyperfine structure. The line observed in D-glucitol has $g = 2.002 \pm 0.002$. The spectrum observed in α-D-xylopyranose is asymmetrical and appears to be composed of a single sharp line with $g = 2.003 \pm 0.001$, which is superimposed on a much broader line.

In Figure 2 the spectra observed for irradiated pentaerythritol and D-mannitol are shown. For each of these samples the spectrum is complex. In both spectra there is evidence that the quartet observed in β-D-fructopyranose is present; however, additional lines which are present prevent the quartet from being identified with certainty. In both samples the apparent positions of lines are indicated in the figures. It is known[13] that the terminal primary hydroxyl groups are points of irradiation attack, and it may be of interest to note that D-mannitol possesses two stereochemically equivalent primary hydroxyl groups and that pentaerythritol possesses four.

Irradiated α-D-mannopyranose and sucrose have also been studied, and the spectra of these sugars are shown in Figure 2. The α-D-mannopyranose spectrum consists of three absorption peaks which can be associated in several ways. If peaks A and B are associated with a hyperfine doublet, then, for this pair of lines, $(A/g\beta) = 12$ gauss and $g = 2.002 \pm 0.001$. Another possibility is that A is a single sharp line with $g = 2.006 \pm 0.001$ and that B and C are lines in a hyperfine triplet with the third line unresolved because of its overlap with line A. If B and C are lines in a hyperfine triplet with possible intensity ratio 1:2:1, then $(A/g\beta)$ is approximately 15 gauss and $g = 1.999 \pm 0.001$. To distinguish between these various possibilities for α-D-mannopyranose, it would be necessary to study the paramagnetic resonance spectrum of this sugar at a higher microwave frequency. The spectrum observed for sucrose is complex, and the possible positions of peaks are indicated in Figure 2. The center of the sucrose spectrum is at $g = 2.002 \pm 0.001$. The complexity of the sucrose spectrum is not too surprising, since the crystal structure of this sugar is complicated. One of the carbon atoms (C4) of the furanose ring is displaced from the plane of the other four atoms. The configuration of both rings is such as to allow the larger attached groups to approach as nearly as possible to the plane of each ring.[14]

Rhamnose is 6-deoxy-mannose, or the former carries a terminal methyl rather than a terminal hydroxymethyl group, with other parts being equal, save for enantiomorphism. The marked difference in their spectra perhaps reflects this structural dissimilarity.

In the carbohydrates thus far discussed, the only nuclei with non-zero nuclear spins were protons. In Figure 1 is shown the magnetic resonance spectrum of irradiated 2-amino-2-deoxy-α-D-glucopyranose hydrochloride (α-D-glucosamine hydrochloride). In addition to protons, α-D-glucosamine hydrochloride contains one nitrogen atom. For N^{14} the nuclear spin $I = 1$. The spectrum of α-D-glucosamine hydrochloride shows five lines with an over-all separation between

outermost lines of 74 gauss. The center of this five-line structure is at $g = 2.001 \pm 0.001$. An analysis of this spectrum indicates that the spectrum consists of five equally spaced lines separated by 18.5 gauss. The spectrum can possibly be interpreted as hyperfine structure due to the electron coupling equally with four protons. Another possibility is that the spectrum is due to the electron coupling equally with two protons and a nitrogen. If the spectrum is actually due to the electron coupling equally with two protons and a nitrogen, the fact that the coupling constant for the proton is identical with the coupling constant for the nitrogen is an unexpected and surprising result. For four protons coupling equally to the electron, the expected intensity ratio of the lines would be 1:4:6:4:1, whereas, for the electron coupling equally to two protons and a nitrogen, the intensity ratio of the lines would be 1:3:4:3:1. One other possibility is that the spectrum is due to the electron coupling equally with two nitrogens, for which case the expected intensity ratio of the lines would be 1:2:3:2:1. To aid in making an estimate of the relative intensities of the five lines in the α-D-glucosamine hydrochloride spectrum, the first derivative record given in Figure 1 was integrated. An estimate of the intensities was made by trying to reproduce the observed spectrum from five lines of equal width but different peak heights. Only a very crude estimate could be made because of the large amount of overlap of the lines, but this crude analysis seemed to indicate that the ratio of the relative intensities was approximately 1:3:4:3:1.

CONCLUSIONS

In most of the irradiated carbohydrates studied, the existence of hyperfine structure in the paramagnetic resonance spectrum suggests the general nature of the radical produced by the radiation. From a consideration of the chemical structure of some of these sugars, some of the similarities observed in the spectra of different sugars are not surprising.

For α-D-glucopyranose monohydrate and α-D-galactopyranose the structures are given in Figure 1. The only difference in structure of these two sugars is the relative configuration about C4. Hence, since there is such a great similarity in the structure of these two sugars, it is not surprising that the radicals produced by irradiation of these molecules would give similar paramagnetic resonance spectra. The spectrum for α-D-mannopyranose is different from that of α-D-glucopyranose monohydrate and α-D-galactopyranose. This would seem to indicate that the configuration of C2 in these sugars influences the type of radical formed.

The spectra of the two ketohexoses, β-D-fructopyranose and L-sorbose (detailed structure unknown), are similar. The spectra of erythritol and D-threitol were found to be similar, and again the only difference in structure of these two is the relative position of a hydroxyl group.

Many questions about the effects of radiation damages to carbohydrates and the free radicals produced are still unanswered. However, it is hoped that the results of the paramagnetic resonance studies which have been carried out can be used in helping to decide what radicals are formed by the ionizing irradiation. On the basis of the present work, further studies seem justified. The study of irradiated single crystals of carbohydrates would probably yield additional information.

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INCREASE IN FITNESS IN EXPERIMENTAL POPULATIONS RESULTING FROM HETEROSIS

BY HAMPTON L. CARSON

DEPARTMENT OF ZOÖLOGY, WASHINGTON UNIVERSITY, ST. LOUIS, MISSOURI

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Introduction—A local population of a species, or deme, consists of an array of individual genotypes. At any one time level, the deme represents the active evolutionary interface between the hereditary material and the environment. Genetic novelties due to new mutations or recombinations are automatically tested when the deme is under natural selection. If a genetic change improves the fitness of its carriers, natural selection may be expected to reproduce the change differentially. The result is adaptive evolution.

The experiments here described represent an attempt to study the process of adaptation directly. The equivalent of one gamete of foreign genetic material is introduced into an experimental population having a size which is held by natural selection in equilibrium with a rigidly controlled and limited environment. Although the environment is unchanged, this introduction is followed by an immediate threefold increase in the population size. Such an increase, occurring as it does under continual strong natural selection, reflects a corresponding increase in the biological efficiency of the group under the specific environmental conditions embodied in the experiment. The evidence is strong that this increase is due to

immediate contributions to fitness made by new heterotic combinations formed in the populations.

Materials and Methods.—Two strains of *Drosophila melanogaster* were used. The first of these, hereafter referred to as "se ss ro," is a laboratory stock homozygous for five third-chromosome recessives. These are: sepia (se), spineless (ss), kidney (k), ebony-sooty (e^s), and rough (ro). The second strain used was the wild-type Oregon-R.

Each experimental population was maintained in a chamber made of an ordinary glass shell vial (95 × 25 mm.) to which a tube of cellulose acetate of the same size and diameter as the shell vial is attached by cellophane tape. The chamber of the latter tube contains a small platform of blotting paper. The unit is closed at the end with cotton. The glass vial contains 9.5 cc. of a carefully prepared uniform cornmeal-Karo-agar medium. Ten grams of granulated agar-agar are dissolved in 200 cc. tap water, then 33 cc. dark Karo (maize) syrup are added. While stirring, 115 gm. of yellow cornmeal (maize; Quaker Oats Co.) mixed with 400 cc. cold water are added. After adding 500 cc. more water, the mixture is boiled for at least 5 minutes or until the total volume is exactly 1,100 cc. Under some atmospheric conditions it is necessary to add a small amount of water after 5 minutes of boiling in order that the final volume will be 1,100 cc. Just before pouring 9.5 cc. of medium into each shell vial, 10 cc. of a 10 per cent solution of Tegosept in 70 per cent alcohol are added. The population food must be made in a small vessel and stirred constantly to keep it uniform in consistency. This food is prepared twice a week and kept under refrigeration. It is prepared for use by yeasting the surface with 10 mg. of Fleishmann's fresh dry yeast, moistened with one drop of distilled water. A strip of absorbent paper toweling, 15 × 75 mm., is doubled and pushed down into the food cake to the bottom of the vial.

Populations are begun in such chambers and a new vial substituted for the old at exact intervals, so that the population of flies in the tube has access to each food cake for 48 hours only. After being exposed to three consecutive such food vials, the entire adult population is etherized lightly, weighed, and counted on the same day and at the same hour each week. The flies are then immediately replaced, together with any newly hatched flies (see below), in the chamber. A clean cellulose acetate "supervial" is used each week; this change is also made on the counting day. The vial with which the population has contact immediately after counting is left in position for 24 hours only. This latter vial is then discarded, or the eggs therein used for samples, and a new cycle of three consecutive 48-hour periods is instituted.

The three 48-hour vials from each week are plugged tightly with cotton and kept separately from the adult population; no further yeast or moisture is added. All young flies hatching from these vials are collected onto unyeasted food at least every 2 days. Twice a week (once in addition to the day on which the population is counted) such accumulated hatching flies are etherized lightly, weighed, counted, and added to the adult population. Vials are not discarded until all flies have emerged. The system thus maintains a closed vial population the size of which is in equilibrium with the food source as soon as the weekly deaths are equal to the weekly additions. The basic design is similar to that used by Buzzati-Traverso,[1] except that in the present case the total mass of the adult population and the young

hatching flies are measured each week. All experiments were carried out at 25.5°C.; the mean generation time under the strong selective conditions is long, approximately 14 days.

The Experiments.—A base control population (C-1, Table 1) of se ss ro was

TABLE 1

SIZE AND PRODUCTION OF EXPERIMENTAL POPULATIONS OF *Drosophila melanogaster* AT EQUILIBRIUM

POPULATION	WEEKS AT EQUILIBRIUM	POPULATION SIZE		PRODUCTION	
		Mean No. Individuals per Week	Mean Wet Weight (Mg. per Week)	Mean No. Individuals per Week	Mean Wet Weight (Mg. per Week)
Controls:					
C-1 se ss k e^s ro Feb.–Oct. '57	32	161.6 ± 6.4	90.3 ± 3.0	100.8 ± 4.4	48.4 ± 1.9
C-2 se ss k e^s ro Dec. '57–Apr. '58	20	158.9 ± 8.4	88.4 ± 2.9	65.5 ± 5.1	29.1 ± 2.4
C-3 se ss k e^s ro Dec. '57–Apr. '58	20	154.4 ± 4.4	88.7 ± 2.0	61.0 ± 3.3	27.6 ± 1.5
C-4 Oregon-R May–Sep. '58	16	380.2 ± 16.1	226.4 ± 8.1	150.5 ± 9.2	84.2 ± 5.2
C-5 Oregon-R May–Sep. '58	16	330.5 ± 17.6	190.4 ± 9.8	151.6 ± 11.0	82.7 ± 6.8
Experimentals:					
E-1 se ss k e^s ro with *n* Oregon autosomes Mar.–Aug. '58	20	457.4 ± 13.7	292.5 ± 9.1	171.0 ± 10.6	91.0 ± 5.9
E-2 se ss k e^s ro with *n* Oregon autosomes Mar.–Aug. '58	20	502.6 ± 14.9	318.6 ± 8.9	201.1 ± 14.4	105.2 ± 8.0

begun on January 2, 1957, using 50 female and 50 male flies from stock. On October 7, 1957, this population was divided into four replicates (C-2,C-3,E-1, and E-2, Table 1). After equilibrium was again reached in these four replicates, one young, wild-type male was introduced into each of the two experimental populations (E-1 and E-2) on December 23, 1957. These males were F_1's from a cross of an Oregon-R male by an se ss ro female taken from the population into which the male was subsequently introduced. The ma'e which was put into population E-1 lived for approximately 21 days; the male in population E-2 lived for 14 days. No flies were introduced into the controls, C-2 and C-3. Control populations C-4 and C-5, made up from Oregon-R stock, were established on February 22, 1958.

Table 1 summarizes the size and production of these seven populations after inspection of the data indicated that the population in question was no longer systematically rising and was thus at approximate equilibrium. Each figure in the table represents the mean size or production of the population over a period of consecutive weeks. Both the mean number of individuals and the mean wet weight, with their respective standard errors, are given. Figures 1 and 2 show the progressive changes in population size (weight) and production (weight), respec-

tively, following the addition of single males to experimental populations E-1 and E-2 (*solid lines*). Figure 3 shows the change in frequency of three of the five types of mutant homozygotes (i.e., se/se, ro/ro, ss/ss) which are formed in the populations following the introduction of the males. The data given in Figure 3 are based on counts of the entire population; the two replicates were essentially similar, and the data have been combined. Free recombination of all third-chromosome genes was observed, and e^s/e^s and k/k homozygotes were likewise present throughout the course of the experiment. Fifteen generations after introduction of their wild-type alleles, all three of the five types of mutant genes which were closely followed remained in the population in substantial frequency, as indicated by the frequency of the homozygous classes observed. Estimates of gene frequencies for these three genes, based on the square roots of the frequency of the homozygous classes after 15 generations, are as follows: ro = 53.4 per cent, se = 25.3 per cent, and ss = 12.3 per cent.

Discussion.—Introduction of a single set of haploid autosomes (and a Y chromosome) derived from Oregon-R stock into equilibrated populations of se ss ro has resulted in a greater than threefold increase in the size of the population. The number of individuals is approximately trebled, whereas the increase in weight is even greater, being about a factor of 3.5. It follows that an increase both in number of individuals and in size of individual organisms has occurred. Comparable increases in production are likewise observed. These changes occur, in the absence of any environmental change, in an explosive fashion in about three generations. The improvement not

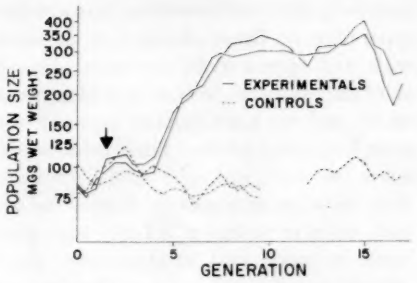

FIG. 1.—Increase in size of two experimental populations of *Drosophila melanogaster* (*solid lines*) following introduction of a single haploid set of autosomes into each of them (*at arrow*).

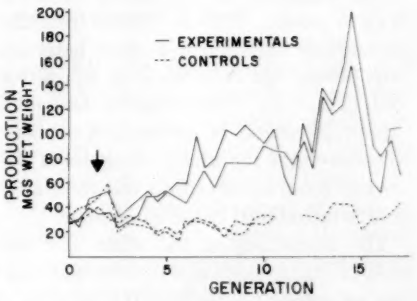

FIG. 2.—Increase in production (milligrams flies wet weight hatched per week) of two populations of *Drosophila melanogaster* (*solid lines*) following introduction of a single haploid set of autosomes into each of them (*arrow*).

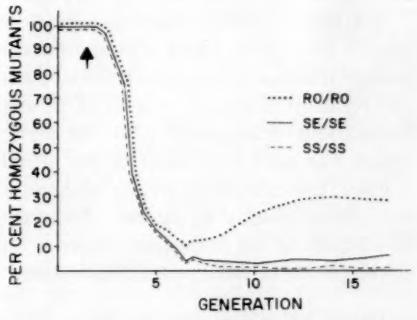

FIG. 3.—Change in frequency of mutant phenotypes in the two experimental populations shown in Figs. 1 and 2. The arrow indicates the point at which the single males were added.

only is quickly achieved but has not been observed to undergo breakdown in 15 generations. These changes in population size and production are concurrent with, and appear to be correlated directly with, a drop in the frequency of the third-chromosome recessives which were being followed. These genes (at least se, ss, and ro) have quickly assumed what appear to be balanced intermediate gene frequency levels. Such behavior is to be expected in at least four of these genes (se, ss, e, and ro). Considerable evidence exists in the literature to suggest that these genes regularly display heterosis in experimental populations, that is, each tends to persist at a fairly high gene frequency, in some cases for 70 generations or more, and is apparently not being progressively eliminated.[2-6] The present experiments indicate that heterosis is not only present but may immediately serve to improve fitness in the population as indicated by the correlated sharp rise in the population size and production (i.e., the biological efficiency of the group).

The improvement found cannot be due to the simple "covering-up" by dominance of the deleterious effects caused by the mutant genes or polygene segments of the se ss ro stock. This is evident from the fact that the polymorphic experimental populations (E-1 and E-2) show heterosis in that they transgress, in their size and thus fitness, not only the se ss ro controls but the donor Oregon-R populations as well (Table 1). The relative contributions of different genes to this heterosis cannot, of course, be ascertained in these experiments. Indeed, there is no reason to believe that the wild-type alleles of the particular oligogenes followed as markers are any more important in this than any of the many other uncontrolled polygene groups introduced in the same male.

The immediacy of the effect and the rapidity of the assumption of a higher level of fitness leads one to conclude that coadaptation could not have entered into this adjustment. Rather, the salutary effects of simple hybrid vigor (= luxuriance) involving possibly a large number of autosomal loci can apparently be immediately exploited by a population and be retained without immediate breakdown. Evidently, natural selection accomplishes this through the prompt development of balanced polymorphism. Whether this heterosis is maintained indefinitely and whether coadaptation may ultimately serve as a basis of further improvement are interesting points to be left to future study of these same populations.

Summary.—Introduction of a single set of haploid autosomes into an experimental population which is equilibrated by natural selection results in an approximately threefold rise in the collective size and production of the population. The environment is rigidly controlled throughout. The biomass of the experimental populations transgresses also the level maintained by that control population which was used as a donor of the introduced wild-type chromosomes. The data suggest that simple heterosis without coadaptation (= luxuriance) may indeed contribute directly to fitness. Natural selection appears not only to prevent the breakdown of the immediate heterosis but also to maintain it through favoring the development of balanced polymorphism.

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*DISSOCIATION OF RUBIDIUM UPTAKE BY NEUROSPORA CRASSA INTO ENTRY AND BINDING PHASES**

BY GABRIEL LESTER AND OSCAR HECHTER

WORCESTER FOUNDATION FOR EXPERIMENTAL BIOLOGY, SHREWSBURY, MASSACHUSETTS

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Introduction.—Previous experiments concerned with the inhibitory effect of deoxycorticosterone (DOC) on the growth of *Neurospora crassa* indicated that this steroid interfered with certain permeability processes, inhibiting the uptake of rubidium, amino acids, or sugars.[1] Since DOC has been shown to inhibit the uptake of a physiologically similar ion—potassium— by other cells and tissues,[2, 3, 4] the phenomenon of rubidium uptake by *N. crassa* has been intensively studied, to provide a basis for elucidating the nature of the effect of DOC. Some of the salient features of this work which have a bearing on the general aspects of cell permeability will be described here; a brief report has been made elsewhere.[5]

This paper presents evidence that the uptake of rubidium by *N. crassa* is an active transport process, which can be dissociated into two discrete events. One is concerned with the entry of rubidium into the cell and the other with the subsequent accumulation of rubidium against an *apparent* concentration gradient. The entry appears to be dependent on metabolic energy but lacks specificity, in that competition between potassium and rubidium is not evident in this phase of uptake. The concentrative accumulation of rubidium in the cell appears to involve a rubidium-binding system which is competitively inhibited by potassium and is blocked by DOC. Although these studies deal with the uptake of rubidium rather than potassium, the usual observation of an equivalence of these ions as nutritional requirements and in their permeability characteristics suggests that the following observations and conclusions apply to potassium as well as rubidium.

Methods and Materials.—The methods and the system employed in this study have been described elsewhere,[1] and they will be only briefly summarized here. Rubidium uptake was examined in a system consisting of washed, germinated conidia suspended in buffer (0.02 *M* NaH_2PO_4 in 0.05 *M* NaCl, adjusted to pH 5.6–5.8 with NaOH). Rubidium^{-85}, admixed with radioactive rubidium^{-86}, was added as the chloride, and the uptake of rubidium was estimated by either or both of two methods: (*a*) by the decrease of radioactivity in the suspending medium after removal of the cells by centrifugation or filtration and (*b*) from the

radioactivity of hot-water extracts of cells rapidly rinsed with buffer while being harvested in a Büchner funnel (very little loss of intracellular rubidium occurred with this treatment); the results obtained by these methods agreed very well with each other. In some experiments, where rubidium-containing cells were desired at the outset, the potassium of the germination medium was replaced with sodium, and rubidium was added as a growth substitute for potassium. Washing with buffer resulted in only a very small loss of rubidium; i.e., washed cells showed a rubidium content approximately that expected on the basis of radioactivity disappearance from the medium. The dry weight of cells in suspension (7–12 mg/ml) was determined for each sample, and the intracellular content of rubidium is expressed as μg. Rb^+ per mg. cell dry weight; no significant changes in cell dry weight occurred during incubation. Calculations of the intracellular concentration of rubidium

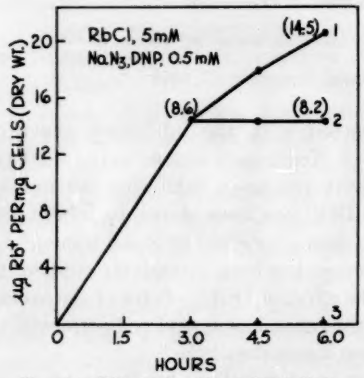

FIG. 1.—Effect of azide and 2,4-dinitrophenol (DNP) on the uptake and retention of rubidium. Curve *1*, control; curve *2*, azide or DNP added at 3 hours' incubation; curve *3*, azide or DNP added 5 minutes prior to the addition of RbCl (time 0). The numbers in parentheses represent the ratios of internal to external rubidium at the associated points.

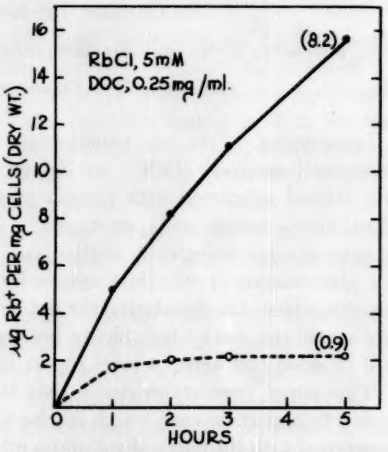

FIG. 2.—Uptake of rubidium in the absence (*solid line*) and presence (*broken line*) of deoxycorticosterone (DOC). The numbers in parentheses represent the ratios of internal to external rubidium at the associated points.

were based on determinations of cell water as 84–85 per cent of the wet weight of filtered cells. Unless otherwise indicated, all the experiments were carried out with 20–30 ml. of suspension in 125-ml. Erlenmeyer flasks, incubated at 30° C. with agitation.

Experimental.—The uptake of rubidium by germinated conidia of *N. crassa* resembles, in certain respects, observations made with potassium (or rubidium) in many other kinds of cells and appears to be a specific, active transport process. Germinated conidia accumulate rubidium to a concentration several times that outside the cell; this accumulation is completely inhibited by 2,4-dinitrophenol (DNP) or azide and is competitively inhibited by potassium. Figure 1 shows the accumulation of rubidium against an apparent concentration gradient and its complete inhibition when DNP or azide are added to a cell suspension almost

simultaneously with rubidium. A unique feature of this system becomes evident when the cells are allowed to take up rubidium for a few hours prior to the addition of DNP or azide. In this case the inhibitors block any further uptake of rubidium, but, more significantly, *an expected efflux of rubidium is not observed.* These observations indicate that the energy requirement for the uptake of rubidium is not the same as that required for the maintenance of intracellular rubidium concentration in excess of that outside the cell.

Another striking characteristic of this system is the unusual stability of the rubidium accumulated by germinated conidia, under conditions where efflux or exchange would be expected. Table 1 indicates the firm retention of rubidium by cells which had been grown in medium where radioactive rubidium replaced the requirement for potassium. These cells were washed and resuspended in buffer

TABLE 1

STABILITY OF INTRACELLULAR RUBIDIUM

Additions to Cell Suspension*	Per Cent Loss of Intracellular Rubidium
Experiment I†	2.5-Hr. Incubation
None	<1
Sucrose (0.03)	1.4
RbCl(0.01 *M*)	3.3
KCl(0.01 *M*)	3.7
Experiment II‡	2.0-Hr. Incubation
Sucrose (0.03 *M*)	1.5
KCl (0.05 *M*)	4.1
Sucrose (0.03 *M*) + KCl(0.05 *M*)	4.8

* The cells were suspended in the buffer described in "Methods."

† Initial intracellular rubidium, 45 μg/mg cell dry wt.; initial cell concentration, 8.5 mg/ml suspension.

‡ Initial intracellular rubidium, 38 μg/mg cell dry wt.; initial cell concentration, 9.7 mg/ml suspension.

with various additions. No significant efflux of rubidium was observed upon incubation in buffer, in the absence or presence of sucrose, which is readily metabolized by these cells. Similarly, incubation in the presence of non-radioactive rubidium or potassium resulted in little efflux of rubidium from these cells, nor did the presence of an energy source, sucrose, lead to an exchange with potassium. These results indicate that, once rubidium enters the cell, it can be maintained against a large apparent concentration gradient, and it is not readily available for exchange with rubidium or potassium entering subsequently. Also, it would appear that the observed uptake of rubidium largely represents a unidirectional flow of rubidium into the cell rather than a two-way flux process.

Having defined certain aspects of rubidium uptake by *N. crassa*, the effect of DOC was examined. The time course of rubidium uptake by germinated conidia in the absence and presence of DOC is illustrated in Figure 2. For a maximal effect the cells were incubated for about 2 hours with DOC before adding rubidium; the control was similarly preincubated without DOC. In the absence of DOC, the rate of rubidium uptake is nearly linear for about 3 hours, and, although the rate diminishes with time, rubidium uptake is still continuing at 9 hours. The presence of DOC reduces the uptake of rubidium, and, characteristically, after about 2 hours no further uptake is observed. In the presence of DOC, the intracellular concentration achieved approximates that of the external rubidium concentration.

This observation suggests that DOC does not prevent the entry of rubidium into the cell but does interfere with the process whereby rubidium is accumulated against an apparent concentration gradient.

This possibility is more clearly indicated in Table 2, which shows the effect of the external rubidium concentration on uptake in the presence and absence of DOC.

TABLE 2

EFFECT OF EXTERNAL RUBIDIUM CONCENTRATION ON UPTAKE IN PRESENCE AND ABSENCE OF DOC

Initial RbCl (mM)	DOC (mg/ml)	Rb^+ Uptake* (μg/mg Cells)	$\frac{[Rb_i]}{[Rb_o]}$
5	...	11.7	6.05
12.5	...	14.6	2.58
25	...	22.8	1.95
5	0.25	2.2	0.93
12.5	0.25	5.1	0.91
25	0.25	11.0	0.90

* 3.0-hr. incubation.

In the absence of DOC the uptake of rubidium increases as the external concentration is increased, but not proportionately. However, in the presence of DOC the uptake of rubidium is directly proportional to the external concentration, and in each case the intracellular concentration approximates that of the concentration of rubidium outside the cell. If, at each concentration of rubidium, the uptake values obtained in the presence of DOC are deducted from those obtained in the absence of DOC, the remainders are found to be quite similar. These results

TABLE 3

EFFECT OF POTASSIUM ON RUBIDIUM UPTAKE

Initial Concentration RbCl (mM)	KCl (mM)	Rb^+ Uptake* (μg/mg Cells)	$\frac{[Rb_i]}{[Rb_o]}$
5	...	14.7	9.1
5	2.5	7.5	3.5
5	5	5.2	2.3
5	10	4.3	1.8
5	20	3.6	1.5

* 3.0-hr. incubation.

TABLE 4

EFFECT OF POTASSIUM ON RUBIDIUM UPTAKE IN PRESENCE AND ABSENCE OF DOC

Initial Concentration RbCl (mM)	KCl (mM)	DOC (mg/ml)	Rb^+ Uptake* (μg/mg Cells)	$\frac{[Rb_i]}{[Rb_o]}$
5	..	...	16.2	9.5
10	..	...	19.6	4.8
5	10	...	5.1	2.2
10	10	...	9.2	2.0
5	..	0.25	2.5	1.03
10	..	0.25	4.7	0.96
5	10	0.25	2.4	0.98
10	10	0.25	5.2	1.06

* 3.0-hr. incubation.

suggest that the capacity of these cells for rubidium in excess of the external concentration is relatively constant. This capacity for rubidium has been found to vary inversely with the potassium content of the cell; however, the sum of intra-

cellular potassium plus the rubidium taken up in excess of the external concentration attains a rather constant maximum value equivalent to about 80 μg. rubidium/mg cell dry weight.[6]

Tables 3 and 4 show the effect of potassium on rubidium uptake in the absence and presence of DOC. The upper half of Table 4 shows that potassium inhibits rubidium uptake in a competitive fashion. In Table 3 it can be seen that the effect of potassium was greater than that expected on the basis of an exact competitive equivalence of potassium and rubidium; for example, with equal concentrations of these ions, rubidium uptake was reduced to about one-third rather than one-half the value obtained in the absence of potassium. It should be noted that as the external concentration of potassium is increased, the uptake or rubidium asymptotically approaches a value corresponding to an intracellular concentration approximating that outside the cell. In other experiments, using still higher potassium concentrations, the uptake of rubidium was about equivalent to that expected on the basis of a passive diffusion of rubidium into the cell.

Table 4 shows that in the presence of DOC, potassium has no inhibitory effect and that the uptake is approximately that expected for a passive diffusion of rubidium. The inability of high concentrations of external potassium to prevent entry of rubidium into the cells and the lack of any competitive effect of potassium on rubidium uptake in the presence of DOC indicate that the entry of rubidium is a relatively non-specific step. On the other hand, the accumulation of rubidium against an apparent concentration gradient appears to be a specific event, as evidenced by the competition between rubidium and potassium.

The data presented so far could suggest that rubidium enters the *Neurospora* cell by a passive process and is then concentrated by some other process. However, it has already been observed (Fig. 1) that DNP or azide practically completely blocks the uptake of rubidium. Since this effect was obtained under conditions where concentration against an apparent gradient could occur and a small uptake might be relatively obscure, the effect of these inhibitors was examined in the presence of DOC. Table 5 shows that DNP or azide almost completely abolishes rubidium uptake under these conditions. These results indicate that the entry of rubidium is not "passive" but is an "active," energy-requiring event under conditions where rubidium does not accumulate against an apparent concentration gradient.

TABLE 5

EFFECT OF AZIDE OR DNP ON RUBIDIUM UPTAKE IN PRESENCE AND ABSENCE OF DOC

Initial Concentration RbCl (mM)	Initial Concentration NaN_3 or DNP (mM)	DOC (mg/ml)	Rb^+ Uptake* (μg/mg Cells)	$\frac{[Rb_i]}{[Rb_o]}$
5	...	...	12.0	6.0
5	0.5	...	0.25	0.1
5	...	0.25	2.5	1.02
10	...	0.25	4.8	0.98
5	0.5	0.25	0.25	0.1
10	0.5	0.25	0.50	0.1

* 3.0-hr. incubation.

The separateness of entry and intracellular accumulation against an apparent gradient can also be demonstrated by the differential effect of temperature on

these phases of rubidium uptake. With an initial external concentration of rubidium of 5 mM, the uptake values in the absence of DOC represent mainly the specific accumulation process, while in the presence of DOC the uptake values represent mainly the entry process. The data given in Figure 3 indicate that the accumulation process is more sensitive to temperature changes than the entry process. Thus, lowering the temperature from 32° to 22° causes a marked decrease in rubidium uptake, in the absence of DOC, whereas a similar temperature change has little effect on the rate of entry, in the presence of DOC. With a further decrease of 10°, the rate of uptake is decreased in both cases, again suggesting that the entry process is also dependent on cellular activities.

Discussion.—The data presented are schematically summarized in Figure 4, where the uptake of rubidium is visualized as a diphasic phenomenon consisting of

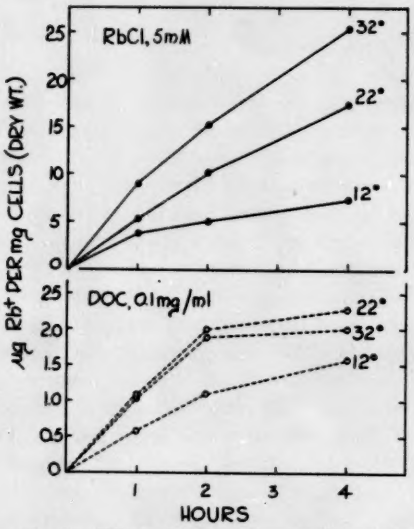

FIG. 3.—Effect of temperature on the uptake of rubidium in the absence (*solid lines*) and presence (*broken lines*) of deoxycorticosterone (DOC). The cell suspensions were incubated at 30° C. for 2 hours with or without DOC prior to the addition of RbCl (time 0) and subsequent incubation at the temperatures indicated. Note the difference in the ordinate scales of the upper and lower portions of this figure.

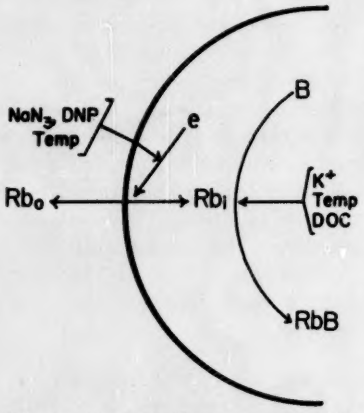

FIG. 4.—A schematic summary of rubidium uptake

entry and an accumulation process involving selective binding. *Neurospora crassa* is considered here as presenting a barrier, perhaps the cell membrane, through which rubidium cannot pass without an expenditure of energy (e). Thus, when the energy supply is reduced, either by a large decrease in temperature or especially by DNP or azide, the entry or rubidium is completely inhibited. Although energy-dependent, the entry of rubidium resembles a passive diffusion process, since rubidium appears to be equally distributed between cell water and the external medium when accumulation is blocked by DOC or high external potassium con-

centration. Moreover, the entry event appears to lack specificity, since potassium does not exhibit any competitive effect on the entry of rubidium, in the presence of DOC. Similarly, when rubidium uptake has been so reduced by high potassium concentrations that the intracellular rubidium concentration approximates that outside the cell, further increases in potassium concentration no longer affect the entry of rubidium. These observations indicate that the competitive interaction between rubidium and potassium observed during uptake probably occurs subsequent to entry, in the accumulation process. The lack of such specificity, in the absence of accumulation, indicates that a transport hypothesis involving the presence of rubidium-potassium specific carrier in the cell membrane is not applicable to the entry phase of rubidium uptake by *N. crassa*.

At present, the mechanism of the entry event is obscure, but any proposals on its nature should take into account its energy-dependence and lack of specificity. These characteristics would obtain for a mechanism such as pinocytosis,[7] which would require energy for membrane movement, resulting in a non-specific transfer of rubidium into the cell; similar features could be envisioned for a contractile-lattice membrane[8] or a contractile process involving an interaction of intracellular and surface proteins.[9] As sole determinants of permeability processes, such proposals have encountered conceptual difficulties in providing for specificity and concentrative features.[10] These difficulties are obviated in the work presented here, since the entry of rubidium lacks specificity, and it can be dissociated from the concentrative event. Thus a mechanical action of the cell surface merits serious consideration as the initial phase in rubidium uptake by *N. crassa* and perphaps as a component of permeability phenomena in other organisms as well.

The process by which rubidium is accumulated in excess of the external concentration is characterized by (*a*) the relatively constant capacity of *N. crassa* for rubidium; (*b*) the maintenance of the internal rubidium concentration in cells incubated in the absence of external rubidium or in the presence of DNP or azide; (*c*) the relatively insignificant exchange between intracellular rubidium and extracellular rubidium or potassium; and (*d*) a competition between potassium and rubidium. These characteristics are not consistent with a hypothesis which assumes flux across the cell membrane in both directions and no differences between the states of intra- and extracellular rubidium or potassium. The operation of a specific pump mechanism, dependent upon metabolic energy for the maintenance of intracellular rubidium against a concentration gradient, is negated by the lack of effect of DNP or azide on the level of accumulated rubidium, although these inhibitors prevent the entry of rubidium.

In view of the above characteristics, the accumulation of rubidium in excess of the external concentration seems best explained by postulating the existence of a specific cytoplasmic system for binding rubidium. This system is assumed to involve a limited number of binding sites (B) which can combine with either rubidium or potassium in a form (RbB or KB) which is not readily dissociated. Since potassium does not readily exchange with rubidium already present in the cell, presumably as RbB, the site of potassium competition would be a specific mechanism responsible for the formation of RbB. The inhibitory effect of DOC on rubidium uptake also appears to involve the binding event, since DOC does not prevent the entry of rubidium. The action of DOC should be directed toward the dissociation

of RbB, its formation, or an alteration of B, but the data do not permit a decision between these possibilities. Similarly, it also remains to be determined whether temperature changes directly affect a specific concentrating system or other related processess. While various cytoplasmic mechanisms have been proposed to account for *K* accumulation in cells,[11–15] the present data are insufficient to decide between these or to formulate other mechanisms. The resolution of this problem in detail awaits the demonstration and characterization of a specific rubidium-binding system in vitro.

Certain features of rubidium uptake in *N. crassa* have more or less close counterparts in other micro-organisms. Thus in *Escherichia coli* there appears to be a limited capacity for potassium accumulation, efflux is not easily effected, nor does intracellular potassium readily exchange with external potassium unless certain carbon sources are present.[11,16] With *Alcaligenes fecalis*, on the other hand, exposure to a potassium-free medium results in an efflux of most of the intracellular potassium; also complete exchange of intracellular potassium readily occurs.[15] The variations in the stability of bound potassium (or rubidium) among these cells raise the possibility that a spectrum of cytoplasmic binding agents (B) exists which have different affinities for potassium. In a given cell type, intracellular potassium might be associated with one or more kinds of B. The postulate that different types of B can coexist in the same cell could explain the heterogeneity of intracellular potassium in kidney,[17] muscle,[18, 19, 20] and other tissues,[21] with respect to exchange or efflux.

Superficially, the uptake of rubidium by *N. crassa* resembles the active transport of potassium in other cells, in that rubidium *seems* to be taken up against a concentration gradient and that this uptake has an energy requirement. However, the evidence presented indicates that two dissociable processes are operative, each exhibiting some, but not all, of the features of the over-all phenomenon. It does not necessarily follow that this description of active transport in *N. crassa* can be applied to other cells. However, these observations may have a general significance by suggesting that attempts to explain permeability phenomena should not be limited to considerations of a single primary mechanism.

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*THE THERMAL CONDUCTIVITY OF CARBON DIOXIDE IN THE REGION OF THE CRITICAL POINT**

BY LESLIE A. GUILDNER†

DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY, CAMBRIDGE, MASSACHUSETTS

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Introduction.—Thermal conductivity phenomena very close to the critical point of CO_2 have not been determined. Reported attempts either have completely skipped the region of most interest or are obviously in error. A new effort based on extensive analysis of the problem was undertaken with equipment exceptionally well suited to the rigorous treatment of the data from which the thermal conductivity can be obtained.[1]

Equipment.—The cell was of the massive concentric cylinder type with a uniform conductivity gap, d, of 0.06955 cm. along the vertical axis and at the bottom. Heat was supplied in a center well of the inner cylinder, and temperature differences were measured by Chromel-P Alumel thermocouples placed at the top and center of the cylinders. A heat-guard at the top was brought to the same temperature as the inner cylinder, and thus heat loss along the thermocouple and heater wires was avoided. The thermocouples were calibrated in place against a platinum resistance thermometer contained in a thermostat bath regulated within ±0.001° C. All voltage measurements were made on an Eppley thermocouple potentiometer, which was carefully calibrated. Pressures were measured on a dead-weight gauge calibrated for the vapor pressure of CO_2 at 0° C., and they were controlled by a special regulator system to about 1 part in 100,000.

Theory.—The energy conducted across length, l, of the annulus, d, between two infinite concentric cylinders is

$$Q = \frac{2\pi K l \Delta T}{\ln r_2/r_1}.$$

The constant for the cell is obtained from this term and a similar term for the bottom,

$$Q = \frac{\pi r_1^2 K}{d} \Delta T,$$

together with two correction terms, one for the corner at the bottom, and another

for the heat-guard gap. By the use of newly developed measurement techniques the cell dimensions were determined with such dependability that the cell constant can be specified within 0.1 per cent.

The problem of convection has been generally recognized by those measuring the thermal conductivity of dense gases. When a flat plate cell is used, it is assumed that no convection occurs if the hotter plate is placed horizontally above the colder, although data obtained in some cases suggest that this may not be true. In other cases, cells have been designed with very narrow annuli to reduce the effect of convection. There is also the criterion that the Grashof-Prandtl product, namely,

$$\text{Gr. Pr} = \frac{d^3 g \alpha \rho^2 c_p \Delta T}{\eta \quad K}, \text{ be less than 1,000.}$$

Here d is the annulus width, g is the acceleration of gravity, α is $-\left(\frac{1}{\rho}\frac{\partial \rho}{\partial T}\right)_p$, ρ is the specific gravity, c_p is the specific heat capacity at constant pressure, η is the viscosity, K is the thermal conductivity at low pressure, and ΔT is the temperature drop across the annulus.

In a more fundamental consideration of convection based on the work of Jones and Furry,[2] it can be shown that, for the annulus between two infinite coaxial cylinders, the ratio of heat transferred by convection to the conductivity is $(q/\Delta T)_{\text{conv}}/\text{K} = \frac{\pi \bar{r}}{720} \cdot \text{Gr. Pr if } d/\bar{r} << 1$. The convection conduction takes place by laminar flow up to a value of Gr. Pr of about 12,000 and, if the temperature rise is not excessive so that the other factors are constant, is linear versus ΔT. Near the critical region, large convective effects are encountered, and they must be eliminated by extrapolation of the apparent thermal conductivity versus ΔT to zero ΔT. If the temperature rise becomes large enough, turbulent convection sets in, with a large increase in slope of the total conductivity versus ΔT, so that the extrapolation must be based on measurements made in the region below the onset of turbulence. As the critical temperature and density are approached closely, the allowable temperature rise becomes smaller. Ultimately, the conduction by laminar convection becomes very large, and the angle between the straight lines of the laminar and turbulent conductivities versus ΔT approaches 180°.

Experimental.—Duplicating the isotherms of the pVT measurements reported by Michels and Michels[3] (M and M) and Michels, Blaisse, and Michels[4] (M B and M), measurements were made on 99.99+ per cent pure CO_2 at average gas temperatures of 32.054°, 34.721°, 40,089°, and 75.260° C. at small density intervals around the critical point. Pressures corresponding to a given density were determined from the data of M and M, and M B and M. Two sets of triplicate measurements of the temperature drop for a preselected heat input were made for both the top and the middle thermocouple. Three different energy inputs were used, so that the temperature rises were in the ratio of 1:2:4. The total conductivities, corrected for asymmetric temperature distribution of the inner cylinder, were extrapolated to zero temperature rise. The loss of heat by radiation and through the supporting pins, determined by measurement of the heat transfer in a vacuum, was deducted to give the final net thermal conductivities.

Results.—Based on the work of M and M, M B and M, and Wentorf,[5] the value of $\rho = 0.474$ gm/cc was used for the critical density. The results in Table 1 are represented by the isotherms of Figure 1, giving the thermal conductivity versus density with special emphasis on the critical region. The values of the conductivity obtained from maximum temperature rises greater than $80\mu V$ (about 2° C.) are precise within 0.1 per cent, and extrapolate linearly within 0.1 per cent. It is expected that such extrapolated values have a total probable error of 0.2 or 0.3 per cent. However, the onset of turbulence occurs at low temperature rises (1–$8\mu V$, corresponding to 0.025°–0.20° C.) near the critical, and in fact, where a $1\mu V$ maximum temperature rise must be used, because of loss of precision and the merging of the two regions graphically, it is not clear that the turbulent region has been avoided. This is true in particular for the values at 32.054° C. and $0.400 < \rho < 0.550$. The maximum temperature rises used for the extrapolation are indicated in column 4 of Table 1. In column 5 the point at which turbulence arose is indicated.

TABLE 1

p (Atm.) (1)	ρ (gm/cc) (2)	$10^3 K$ I.T. cal cm^{-1} sec^{-1} deg^{-1} (3)	Max. ΔT (μV) (4)	Onset of Turb. ΔT (μV) (5)	Probable Error (Per Cent) (6)
		30.90° C. $dE/dT = 42.45\mu V$/deg			
2.18	0.0036	3.99	228	..	0.3
30.82	0.0651	4.60	128	..	0.3
57.62	0.161	6.37	67	80	0.4
		32.054° C. $dE/dT = 42.68\mu V$/deg			
72.51	0.300	12.6	6	7	1.5
74.29	0.400	31	1	..	5
74.49	0.450	57	1	..	5
74.56	0.476	70	2.3	..	5
74.62	0.500	49	1	..	5
74.99	0.550	28	1.7	2	5
75.90	0.600	21.5	2.6	3.5	3
79.67	0.650	19.4	7	..	2
		34.721° C. $dE/dT = 42.79\mu V$/deg			
78.18	0.400	20	4	5.3	5
79.13	0.474	25	4	..	5
80.46	0.550	19.6	8	..	2
		40.087° C. $dE/dT = 42.99\mu V$/deg			
85.81	0.400	15.1	6	..	1.5
88.91	0.474	16.5	2	..	3
91.85	0.550	17.5	9	10.5	1
		40.107° C. $dE/dT = 42.99\mu V$/deg			
124.9	0.737	19.7	32	40	0.5
195.2	0.840	23.14	95	..	0.3
		75.26° C. $dE/dT = 43.22\mu V$/deg			
150.7	0.474	13.00	40 *	45	0.5
178.2	0.575	15.48	38	50	0.3
300.0	0.770	20.68	..	..	.. *

* Tentative.

By the development of special measuring techniques and extreme experimental precautions, it was possible even on the lowest temperature rises of $0.25\mu V$ to obtain a precision of ±5 per cent (i.e., ±0.0003° C.), and thus the data while not as good as could be wished, are significant. The uncertainty about the existence of turbulent convection for these measurements is unfortunate, but if there is

error for this source, it is to cause the highest values of each isotherm to be too low.

Discussion.—Sellschopp,[6] Lenoir and Comings,[7] and Timrot and Oskolkova[8] have reported measurements of the thermal conductivities of dense CO_2 covering the range of densities considered in this research. However, only Lenoir and Comings have any measurements in the region of ρ_{crit}. Sellschopp's results, which agree quite well for $0 < \rho < 0.3$ and $0.7 < \rho < 0.9$, appear to reflect unextrapolated

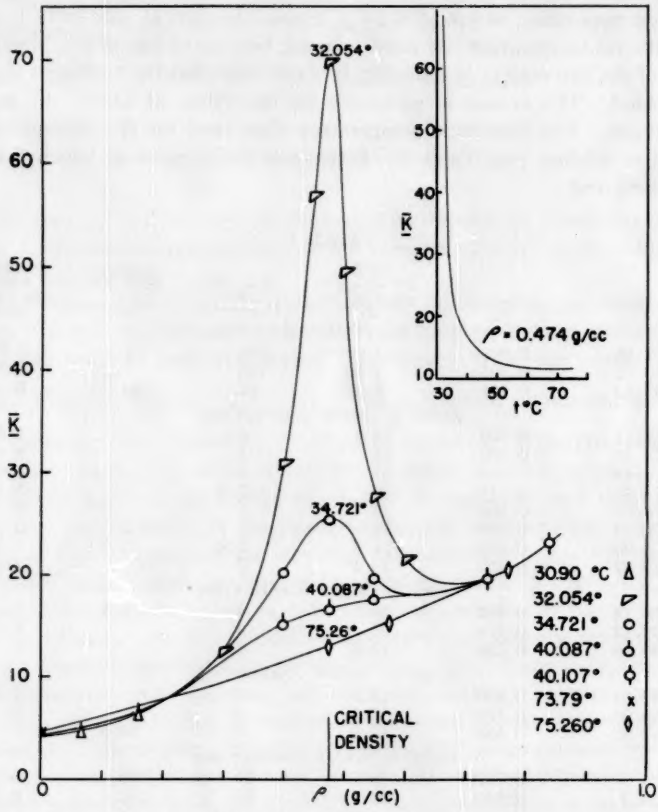

FIG. 1. Isotherms of the thermal conductivity of CO_2 versus density. $K = 10^5$ I.T. cal. cm.$^{-1}$ sec.$^{-1}$ deg. C.$^{-1}$, ρ = gm/cc.

convection in the range $0.575 < \rho < 0.7$ and at $\rho = 0.375$ and do not cover the region of higher interest, $0.375 < \rho < 0.575$. Lenoir and Comings measured the thermal conductivity at 41.11°, 56.67°, and 67.22° C. In the critical region their five points are scattered but are in approximate agreement with the 40.09° and 75.26° isotherms. Timrot and Oskolkova, measuring with a hot-wire cell having a very small surrounding tube (I.D. of 0.84 mm.), recognized the convection problem and made approximate corrections on certain of their density-temperature points.

As well as can be estimated, on their approach to the critical point (approximately 33° C. and 75 kg/cm², and 35.5° C. and 80 kg/cm²), they obtained a thermal conductivity of 11.1 cal cm^{-1} sec^{-1} deg^{-1} at $\rho = 0.28$ and 10.0 at $\rho = 0.33$. This agrees with the results obtained in this work within experimental error in the first case and is about 21 per cent low in the second case. No data fall within the range $32 < t < 40$ and $0.35 < \rho < 0.60$.

Summary.—By the control of convection through extremely careful experimental procedures, it has been shown that the thermal conductivity of carbon dioxide in the density range near the critical density of 0.474 gm/cc exhibits marked increases as the temperature of the critical point is approached from higher temperatures. The isotherms of thermal conductivity versus density are nearly symmetrical about the critical density, although some additional increase is observed on the low-density side. As can be seen in Figure 1 from the inset of the isometric of K versus t, the rate of increase of thermal conductivity with diminishing temperature at constant density, $-\left(\frac{\partial K}{\partial T}\right)_\rho$, suggests an extremely high, if not infinite, value of the thermal conductivity at the critical point itself. At temperatures higher than 40° C. above the critical temperature, the shape of the isotherm is nearly "normal," and at low and high densities the isotherms near the critical temperature behave in a normal fashion also.

A more extensive paper covering this material is in preparation and will be offered for publication shortly.

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† Staff member, Division of Sponsored Research of the Massachusetts Institute of Technology.

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INFORMATION TO CONTRIBUTORS

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